Opportunistic pathogens of the normal human microbiota

Sheila Patrick BSc PhD (Edin)

DSc

The University of Edinburgh

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Declaration

I hereby declare that the publications on which I am sole author are my own work and that I was the Principal Investigator in relation to the multi-authored publications with the exception of:

Reid JH and Patrick S. 1984. Phagocytic and serum killing of capsulate and non-capsulate *Bacteroides fragilis*. Journal of Medical Microbiology 17, 247-257.

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Connery N, Thompson AS, Patrick S, Larkin MJ. 2002. Studies of *Microthrix parvicella in situ* and in laboratory culture: production and use of specific antibodies. Water Science and Technology 46, 115-118.

where I was a collaborating author with a significant contribution to the work and writing up of the material;

Brook, I, Gerard A, Lambe DW, Maclaren DM, Patrick S and Sebald M. 1990. Pathogenie des infections a bacteries anaerobies. Medecine et Maladies Infectieuses 20 hors serie, 45-47

which is a report of a Round Table discussion held at the International Congress for Infectious Diseases in Montreal 1990, in which I was a participant and

Luczak M, Obuch-Woszczatynski P, Pituch H, Leszcynski P, Martirosian G, Patrick S, Poxton I, Wintermans RGF, Dubreuil L, Meisel-Mikolajczyk F. 2001. Search for enterotoxin gene in *Bacteroides fragilis* strains isolated from clinical specimens in Poland, Great Britain, the Netherlands and France. Medical Science Monitor 7, 222-225,

for which where I contributed *Bacteroides fragilis* clinical isolates.

S. Patrick 17th March 2006

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Abstract

To the colonising bacterium, the human body represents a number of ecological niches, some of which it could be argued are as hostile to the coloniser as even the most extreme of *ex vivo* environments; by the activities of the immune system, these living host niches are actively dedicated to the prevention of their colonisation. Paradoxically, the normal human microbiota of each human extends in estimated total number to approximately 10^{14} per human. This thesis is a compilation of published work that focuses on two anaerobic bacteria of the normal human microbiota: *Bacteroides fragilis* predominantly found in the large intestine; and *Propionibacterium acnes* predominantly found in the skin microbiota. When given the opportunity these bacteria can cross the divide between commensal and pathogen and cause infection. The papers included in this thesis address aspects of the characteristics of these bacteria that may relate to virulence and the association of these bacteria with clinical infection.

Overview

To the colonising bacterium, the human body represents a number of ecological niches, some of which it could be argued are as hostile to the coloniser as even the most extreme of *ex vivo* environments; by the activities of the immune system, these living host niches are actively dedicated to the prevention of their colonisation. Paradoxically, the normal human microbiota of each human extends in estimated total number to more than the number of people that currently colonise the earth; approximately 10^{14} per human. This thesis is focussed upon two quite different bacteria of the normal human microbiota, *Bacteroides fragilis* from the large intestine and *Propionibacterium acnes* from the skin that, when given the opportunity, can cross the divide between commensal and pathogen and cause infection

My interest in this area started in 1981 when as a post-doctoral researcher, I took up a position with Professor Bob Gillies, Chair of Clinical Bacteriology at Queen's University Belfast (QUB). Despite the recognition of the importance of obligately anaerobic bacteria in human infection in the late 1890s, it was not until the 1970s that the recognition of the need for treatment of obligate anaerobes, and indeed for prophylactic antibiotic cover to be inclusive of anaerobes for lower abdominal surgery, became widespread amongst clinicians. Professor Gerry Collee of the University of Edinburgh, from where Professor Gillies had moved to QUB, was one of the Clinical Bacteriologists who fully recognised the potential impact of anaerobic bacteria in relation to morbidity and mortality. Sadly Professor Gillies died suddenly in 1983.

The study of the potential virulence determinants of the obligately anaerobic bacterium most frequently isolated from clinical infection, *B. fragilis*, was in its infancy. As a member of the normal faecal microbiota *B. fragilis* is in the minority when compared with other species, such as *B. thetaiotaomicron* and *B. vulgatus*. At that time there were conflicting reports in the literature concerning both the production and significance of the production of capsules by *B. fragilis* and other *Bacteroides* spp. Prior to my arrival in the Department of Microbiology and Immunobiology at QUB, John Reid, a PhD student had successfully carried out some electron microscopy (EM) on *Bacteroides* spp, including *B. fragilis* American Type Culture Collection (ATCC) 23745,

with the help of Dr Evelyn Dermott, a virologist and cell biologist at QUB. This strain was favoured by many of the workers at that time, particularly in the USA. My background in general Microbiology from Edinburgh University as an undergraduate and in microbial ecology as a post-graduate, under the supervision of Professor John Holding, meant that I had insight into the importance of nutrient availability in relation to bacterial polysaccharide expression. In particular, as a result of the both being taught by and having read Professor Ian Sutherland's book 'Surface Carbohydrates of the Prokaryotic Cell, 1977, London: Academic Press' I searched the literature for a recipe for a defined medium that would sustain the growth of *B. fragilis*, with a view to determining the effects of altered glucose concentration on extracellular polysaccharide production. I also applied capsule staining techniques used during my PhD studies, initially at the University of Edinburgh and latterly at QUB when John Holding moved there during the final year of my PhD studies. I obtained the type strain National Collection of Type Cultures (NCTC) 9343, which had been isolated from a peritoneal abscess in 1955 at St Bartholomew's Hospital, considering that it might be better representative of *B. fragilis* than ATCC23745 which was originally isolated from pleural fluid. Growth in the defined medium immediately revealed within strain capsule variation, which I then discovered had also been reported in the literature for *B. thetaiotaomicron*. I developed a simple step density gradient technique using Percoll, which enabled the separation and enrichment of populations with different sizes of capsules (Patrick and Reid 1983; p13). The mechanism generating the phase variation was unknown and the subject of much debate with a colleague at that time, a newly appointed lecturer in the Department of Microbiology and Immunobiology, now Professor Michael Larkin, who had an interest in bacterial recombination. Lysogenic phage had been suggested as the underlying cause in *B. thetaiotaomicron*, but Michael Larkin introduced me to the literature relating to H1 and H2 flagellar variation in *Salmonella typhimurium*. A DNA inversion mechanism similar to that of *Salmonella* seemed the neater hypothesis, particularly as the phase switch was clearly reversible and reached equilibrium on subculture. I included this in a summary report that I prepared for Professor Gillies in 1981 (Appendix A; p53). The determination of the genetic basis of the phase variation remained elusive and experiments were focussed on studies of the enriched populations in relation to the

potential role of the capsules in virulence. The recognition of the importance of studying pathogenic bacteria *in vivo* was beginning to spread, not least as a result of the influence of Professor Harrry Smith at the University of Birmingham. I therefore compared the growth of the different populations *in vivo*, by using my adaptation for anaerobic bacteria of a model developed by Professor John Arbuthnott and Professor Gordon Dougan that I had learnt during a visit to Trinity College Dublin. ATCC 23745, grew unpredictably *in vivo*, whereas with NCTC 9343 I obtained highly reproducible growth curves *in vivo* (Patrick, Reid and Larkin 1984; p14). Why ATCC 23745 grew erratically has yet to be resolved. In parallel, I carried out joint *in vitro* studies of phagocytosis and serum killing with John Reid (Reid and Patrick 1984; p15) and subjected the different capsular types to extensive EM with some technical help from Alan Coffey (Patrick, Reid and Coffey 1986; p17) The EM revealed that the non-capsulate population had a marginal electron dense encapsulating layer capsule only visible by EM.

An attempt to detect IS1 from *E. coli* K12 in *B. fragilis* in a project jointly supervised by Michael Larkin and involving an Honours project student, Garry Blakely and Michael Larkin's PhD student, Ross Williams proved to be fruitless, suggesting a lack of stable genetic exchange with intestinal *E. coli* and indicating the likelihood of quite distant taxonomic phylogeny, although it did tell us that NCTC 9343 contained a plasmid (Larkin *et al.* 1986; p18).

A novel technique that had recently been introduced into the Department by Dr A Trudgett on his return from a post-doctoral period at the National Institutes for Health in Washington DC, was monoclonal antibody (MAb) production. Several fusions yielded antibodies of disappointing usefulness, which was frustrating, as immunofluorescence microsocopy (IFM) had been developed in the Department by Dr Praful Shirodaria and immunogold EM by Evelyn Dermott. A serendipitous meeting with Professor S Tabaqchali in the queue for the ladies toilets at a Royal Society Discussion meeting in London led to a Wellcome Trust application for funding being obtained for a post-doctoral position for John Reid at St Bartholomew's Hospital. Finally we produced one stable antibody producing hybridoma in Belfast and intriguingly the antibody only labelled some of the bacteria in the population (Reid *et al* 1985; p16).

John Reid tried to produce further MAbs and carried out PAGE and immunoblotting at St Bartholomew's Hospital, while I carried out the IFM and immunogold EM on *in vitro* and *in vivo* grown populations. These experiments yielded a further antibody, which indicated that one of the capsulate types, the small capsule, was antigenically distinct (Reid, Patrick and Tabaqchali 1987; p16).

My continued studies of capsule expression in relation to virulence indicated that the large capsule was not necessary for the survival of *B. fragilis in vivo*, even in the presence of actively phagocytic cells and that the non-capsulate/EDL population haemagglutinated, but not the large or small capsule. Disappointingly there was no evidence for the induction of outer membrane proteins (OMP) such as the iron repressible OMP of *E. coli* when the bacteria were grown *in vivo,* although the results of an Honours Project student suggested that under haem limitation there was induction of an approximately 40 kDa OMP (Patrick 1988, Larkin, McGuigan and Patrick 1988, Patrick *et al.* 1988, Patrick and Lutton 1990; pp 20-22,24). A collaboration with Dr Joop van Doorn of the Free University of Amsterdam, initiated as result of meeting him at the International Union of Microbiology Societies Congress in Manchester in 1986, indicated that there was within strain variation in expression of a fimbrial antigen (Lutton *et al.* 1989; p23). Professor M Emmerson replaced Professor Gillies in the chair of Bacteriology from 1985-1989. He agreed to be named as Principal Investigator on my application to the Medical Research Council (MRC) to obtain personal funding to continue the *in vivo* studies and for the development of bacterial flow cytometric analyses of the antibody labelling. I was subsequently able to employ Debbie Lutton on the remainder of the MRC funding when I was appointed as a Research Officer with Dr Tom McNeill, Consulant/Senior Lecturer in Immunology.

With a new fusion partner and slightly modified protocol, Debbie Lutton and I generated 30 stable hybridoma cell lines secreting antibody specific for surface components of *B. fragilis*. The labelling patterns observed with these MAbs demonstrated beyond any doubt that not only was there phase variation with respect to capsule expression but that there was antigenic variation of surface polysaccharides within populations enriched for either the EDL or large capsule. Dr Alastair Crockard of the Regional Immunology Laboratory allowed us to run bacteria through his Flow

Cytometer, which enabled the analyses of large numbers of bacteria. I first reported these results as in invited speaker at the International Congress for Infectious Diseases held in Montreal in 1990 where I was also invited to participate in a Round Table discussion on the Pathogenesis of Anaerobic Bacterial Infection, held in English, but translated into French by Y Pean and Y Buisson (Patrick and Lutton 1990, Brook *et al.* 1990, Lutton *et al.* 1991, Patrick 1993, Patrick and Larkin 1993; pp25-29). Further MAbs produced during Kevin Wilson's project also indicated that there were antigenically variable surface proteins. The underlying genetic mechanism generating the variation remained unknown, but it was clear that antigenic variation of surface polysaccharides also occurred during *in vivo* growth (Patrick, Lutton and Crockard 1995; p. 30). Data generated by a post-graduate student, James McKenna and intercalated Medical Student, Seamus O'Hagan, gave further insights into the haemagglutination of *B. fragilis* (Patrick *et al.* 1996; p33). Over the same period, Linda Stewart, a Medical Laboratory Scientific Officer at Craigavon Area Hospital where Dr Nizam Damani was the Consultant Bacteriologist, carried out her PhD under the joint supervision of myself and Michael Larkin in which she examined pus and blood culture isolates as well as pus samples directly, by IFM. In addition we prepared polyclonal antisera specific for the common antigen of *B. fragilis*, previously identified by Professor Ian Poxton and Mr Bob Brown at the University of Edinburgh, using some of their original sera. Using this polyclonal antiserum the study clearly showed an improvement in detection of *B. fragilis* by use of IFM when compared with culture (Patrick *et al.* 1995, Patrick 1997; pp31, 35). Sadly Dr McNeill suffered from continued poor health and retired early in 1995.

I provided clinical isolates for a survey of the *B. fragilis* enterotoxin gene carried out by Professor Meisel-Mikolajczyk and colleagues at the Medical University of Warsaw, Poland (Luczak *et al.* 2001; p42). I also explored the possibility for combining IFM with fluorescent *in situ* hybridisation (FISH) using 16S rRNA probes with an Honours project student Simon Houston. This work was completed by a post-graduate student Gordon Ramage and clearly highlighted the limitations of FISH where capsular polysaccharides are present (Ramage, Patrick and Houston 1998; p36). IFM based detection was extended to the bacterium *Microthrix parvicella*, which is involved in the bulking of sludge in waste treatment, in collaboration with Michael Larkin, a PhD student

Nicola Connery and post-doctoral research assistant Dr Andrew Thompson (Connery *et al.*, 2002; p43).

From 1988 I had been contributing lectures in Honours level courses on Bacterial Genetics and Immunology in relation to pathogenic bacteria and in 1990 I initiated an Honours level course on Bacterial Pathogenesis. I became acutely aware that there was no suitable text that combined molecular aspects of bacterial pathogenesis, immunology and the relevant aspects of bacterial genetics. J Wiley and sons offered Michael Larkin and myself a contract to produce such a text and this was published in 1995, Michael Larkin contributing the majority of Chapter 9 (Patrick and Larkin 1995; p32).

In 1995 Mr James Nixon, Consultant Orthopaedic Surgeon at Musgrave Park Hospital initiated a meeting with myself and Professor Sean Gorman of the School of Pharmacy at QUB to discuss possible reasons for the failure of prosthetic hip implants. He specialised in the revision of failed implants and was concerned that there were occasions when the routine diagnostic bacteriology laboratory reported 'no significant growth' when he thought that there might be infection. As a result I was Principal investigator on two successive Arthritis Research Campaign grants with Sean Gorman and James Nixon to investigate the potential role of anaerobic bacteria in failed hip replacements and the application IFM- based detection methods. As a result of hearing of the work of Dr Martin Curran in the Regional Histocompatability and Immunogenetics Laboratory at Belfast City Hospital on the detection of 16S ribosomal DNA, I set up a collaboration to enable a comparison with the IFM detection methods. Dr Michael Tunney was employed as the post-doctoral researcher on the grants and my post-graduate students Gordon Ramage, Donna Hanna and Rebecca Perera contributed to the work. I also initiated a collaboration with Dr Richard Davis and Dr Neil Anderson in the Pathology Department at the Royal Victoria Hospital in relation to the histopathological analysis of tissue samples from sites adjacent to the retrieved implants.

The data from these studies confirmed the involvement of *Staphylococcus* spp of the normal microbiota, such as *S. epidermidis*, and also clearly implicated *P. acnes* as a major contributor to the failure of prosthetic joints. In addition they indicated that many of the isolates were resistant to the antibiotics in current use (Tunney *et al.* 1998a and

b,1999 a and b; Ramage *et al.* 2003; pp37-40,46). Subsequently this work was extended by a PhD student, Josephine Glenn, and Orthopaedic Surgical Trainees Seamus O'Hagan, Michael McMullan and Greg McLorinan. As a result of report of a link between *P. acnes* and sciatica, determined by culture of clinical samples, Greg McLorinan applied both culture and non-culture methods to samples taken during spinal surgery. The link between *P. acnes* and sciatica was not proven, but his data indicated that up to 30% of the surgical wound sites in his study were contaminated by bacteria of the skin microbiota (McLorinan *et al.* 2005; p47).

The pathogenic potential of *P. acnes* remains a key question and I obtained funding from the Research and Development Office of the Health and Personal Social Services Northern Ireland (HPSSNI) and subsequently from the British Orthopaedic Wishbone Trust to examine potential virulence determinants. A post-graduate student, Susanna Valanne, embarked on the study of an abundant surface associated and secreted protein that was evident in serotype II *P. acnes*, but not type I. A Material Transfer Agreement that I negotiated with the former Corixa Corporation in the USA, gave us access to a complete genome sequence of *P. acnes* and led to the identification of this protein as one of a family of five co-haemolysins (Valanne *et al.* 2005; p48). The immuno-purification of the protein was carried out in collaboration with Dr Brian Wisdom of Biology and Biochemistry at QUB, Dr Derek Fairley of the QUESTOR centre at QUB helped with the initial phylogenetic analyses and Gisli Einnarsson provided some technical assistance. Dr Andrew McDowell joined my laboratory as a post-doctoral researcher employed on a 5 year programme grant from the HPSSNI to develop the non-culture detection methods for hip prosthesis to a level where they could be readily adopted in the diagnostic laboratory (McDowell and Patrick 2005; p49). His interest in bacterial phylogeny sparked further investigation of the *P. acnes* sub-types, complementing the MAb typing with sequencing of 16S ribosomal RNA and *recA* genes. These data explained some of the anomalies in the typing and resulted in the identification of a sub-group within the type I *P. acnes* (McDowell *et al.* 2005; p50).

During the period from 1999-2001 when Professor Mark Pallen held the Chair of Microbiology at QUB I was appointed Senior Lecturer in Bacteriology. Mark Pallen's

boundless enthusiasm and interest in complete sequencing of bacterial genomes alerted me to the Wellcome Trust Beowulf funding initiative. As result of my expression of interest in relation to sequencing the *B. fragilis* genome, I was invited onto the strain selection committee and to nominate other members and therefore suggested Ian Poxton from Edinburgh University and Professor Brian Duerden from the University of Wales. I proposed that the type strain NCTC 9343 should be sequenced, as this exhibited the complete range of capsular variants and within strain antigenic variation. The Wellcome Trust staff contacted a number of researchers outside the UK, who I had suggested, with respect to strain selection. My choice of NCTC 9343 proved to be controversial, a number of workers in the USA favoured the rifampicin resistant mutant strain 638R as it was more amenable to genetic manipulation by electroporation. When I then wrote the application to the Wellcome Trust for funding for the project, to be carried out at the Wellcome Sanger Institute in the Pathogen Sequencing unit, I requested funding for sequencing of both strains, and this was subsequently forthcoming. I sent the DNA to the Sanger Institute where the *B. fragilis* team, lead by Professor Julian Parkhill, embarked on the process of shot-gun sequencing, assembly of the sequence and finally the annotation of the putative coding sequences, Dr Anna Cerdeño-Tárraga being mainly responsible for the initial annotation.

I had isolated the DNA from an early low passage sub-culture of NCTC 9343 obtained from the NCTC which I had enriched for the EDL but which was antigenically mixed, in the hope that the sequence might provide some clue as to the mechanism generating the variation. This strategy was successful as in 2001 Julian Parkhill, phoned me to say that there were an unusually high number of regions of DNA present in two alternative orientations. This indicated that specific inversions of these sequences had been occurring at high frequency in the clonal population from which I had isolated the DNA. Seven of these 'fragilis invertible regions' were upstream of O-antigen capsule-like biosynthesis operons and contained the consensus *B. fragilis* promoter sequence. When I looked at the sequences of the inverted repeats that flanked these invertible regions I realised that they were strikingly similar to those flanking the Hin invertible region of *Salmonella* that I had speculated might be involved in controlling capsular variation in 1981 (Appendix A; p53). Lisa Douglas, one of my PhD students at that time

of who had been trying to develop a gene knock-out system for *B. fragilis* in collaboration with Dr Martin Collins in Agriculture and Food Science at QUB, investigated the orientation of the invertible promoters in *B. fragilis* populations enriched for one type of antigenic surface polysaccharide. Dr Garry Blakely was now a lecturer at Edinburgh University, having completed his PhD with Michael Larkin and spent a number years as a Post-doctoral researches at Oxford University, he had considerable expertise in bacterial recombination. I therefore arranged for Lisa Douglas to spend time in his laboratory in Edinburgh where she confirmed the interaction of a putative invertase enzyme with similarity to the *Salmonella* Hin invertase and present in the NCTC9343 genome, with the inverted repeat region along with Garry Blakely's student Matylda Sczaniecka (Patrick *et al.* 2003; p45). The complete genome sequence was published in 2005 and, in addition to the Sanger Institute *B. fragilis* pathogen sequencing team and members of the strain selection committee, involved Dr Val Abbrat from the University of Cape Town South Africa, with whom I had also been collaborating, and Garry Blakely in relation to the interpretation of the sequence data in relation to DNA repair and recombination (Cerdeño-Tárraga *et al.* 2005; p51).

I was invited to contribute a chapter on *Bacteroides* in three volume text Molecular Medical Microbiology (Patrick 2001; p44) and update Brian Duerden's Chapter on Gram-negative non-spore forming obligate anaerobes for the second edition of Principles and Practice of Clinical Bacteriology (Patrick and Duerden 2006; p52).

Complete genome sequence information has brought about a revolution in both the manner in which we are able to study bacteria and also the speed with which we can generate data; with respect to both *B. fragilis* and *P. acnes* it opens up the possibility of in depth analyses of the interactions of these bacteria with the human host as both commensals and pathogens. The challenge for the future will be the application of this knowledge to novel means for the prevention, detection and treatment of bacterial infection.

Patrick S and Reid JH.
1983.
Separation of capsulate and non-capsulate *Bacteroides fragilis* on a discontinuous density gradient.
Journal of Medical Microbiology 16, 239-241.

J. MED. MICROBIOL.—VOL. 16 (1983) 239–241

SEPARATION OF CAPSULATE AND NON-CAPSULATE *BACTEROIDES FRAGILIS* ON A DISCONTINUOUS DENSITY GRADIENT

SHEILA PATRICK AND J. H. REID

Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN

SUMMARY. A discontinuous density gradient procedure was used successfully to separate capsulate and non-capsulate cells of strains of *Bacteroides fragilis*. Bacteria from the capsulate fraction retained their capsules on subculture, but non-capsulate cells reverted to the mixed phenotype.

INTRODUCTION

Considerable emphasis has been placed on the importance of the polysaccharide capsule of *Bacteroides fragilis* as a virulence determinant (Kasper *et al.*, 1977). In studies with rats, the polysaccharide capsule has been shown to promote the formation of intra-abdominal abscesses similar to those found in human infection (Onderdonk *et al.*, 1977).

A homogeneous population of bacteria is necessary to investigate the selective advantage of the capsule in pathogenesis. Variation in the proportion of capsulate and non-capsulate cells within individual *Bacteroides* cultures (Babb and Cummins, 1978) and instability of capsule production by *B. thetaiotaomicron* (Burt *et al.*, 1978) have been reported. Booth *et al.* (1979) reported that capsulate *B. fragilis* were resistant to bacteriophage infection and suggested that this might be used to obtain a capsulate population. Percoll density gradients have been used for the separation of prokaryotic cells, for example, to synchronise cell division (Dwek, Kobrin and Grossman, 1980) and to separate *Escherichia coli* possessing colonisation factor antigen 1 (Giesa *et al.*, 1982). In this paper we report the use of a discontinuous Percoll density gradient to separate capsulate and non-capsulate *B. fragilis*.

MATERIALS AND METHODS

Bacterial strains. *B. fragilis* NCTC9343 was supplied by the Department of Bacteriology, University of Edinburgh Medical School and *B. fragilis* ATCC23745 by the American Type Culture Collection, Rockville, MD. *B. fragilis* NCTC10584 was a departmental stock culture.

Culture methods. Bacteria were grown in defined broth (van Tassell and Wilkins, 1978), the basal medium of Deacon, Duerden and Holbrook (1978) or on plates of lysed human-blood agar (LHBA). Cultures were incubated at 37°C in an atmosphere of 90% H_2 and 10% CO_2 in anaerobic jars with two catalyst sachets per jar.

Microscopy. The presence of capsules was determined by light microscopy with Indian ink and eosin-carbol fuchsin negative staining (Cruickshank, 1965). The proportion of capsulate cells was determined by random field counts of eosin-carbol fuchsin stained preparations under a × 100 oil-immersion objective. All determinations were done with late exponential-phase cultures containing approximately 10^8 cfu/ml.

Density gradient centrifugation. Percoll (a colloidal sol of polyvinylpyrrolidone-coated silica particles; Pharmacia Fine Chemicals Inc., London) is supplied sterile. A stock solution, isosmotic with physiological saline, was prepared by diluting with 1·5 M NaCl in a ratio of 9:1. The *p*H of this solution was adjusted to 7·0 with 1 M HCl. Solutions of 20, 40, 60 and 80% Percoll were prepared by further dilution with 0·15 M NaCl.

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A 2-ml volume of each of these solutions was layered into a test tube to produce a step gradient with 80% Percoll at the bottom and the 20% solution at the top. A broth culture of the test organism (2·5 ml) was applied to the top of the 20% layer and the gradient centrifuged at 2600 *g* for 20 min in a bench centrifuge. After centrifugation the bands were removed with sterile 2-ml syringes fitted with long needles. Bacteria from the gradients were subcultured without further processing.

RESULTS

Approx. 17% of the cells in the *B. fragilis* ATCC23745 culture produced capsules greater than the width of the cell in size when grown in the defined medium of van Tassel and Wilkins (1978). Capsules of the same dimension were observed in ruthenium red stained preparations by electron microscopy. Fewer than 1% of the cells of *B. fragilis* strains NCTC10584 and NCTC9343 produced capsules of similar size when grown under the same conditions. Capsules produced in the basal medium or on LHBA plates were smaller and difficult to assess because the eosin-carbol fuchsin stain tended to shrink from the edge of the cell to produce a bright halo similar to that of a small capsule.

After centrifugation of cultures in defined medium on a Percoll density gradient, cells with

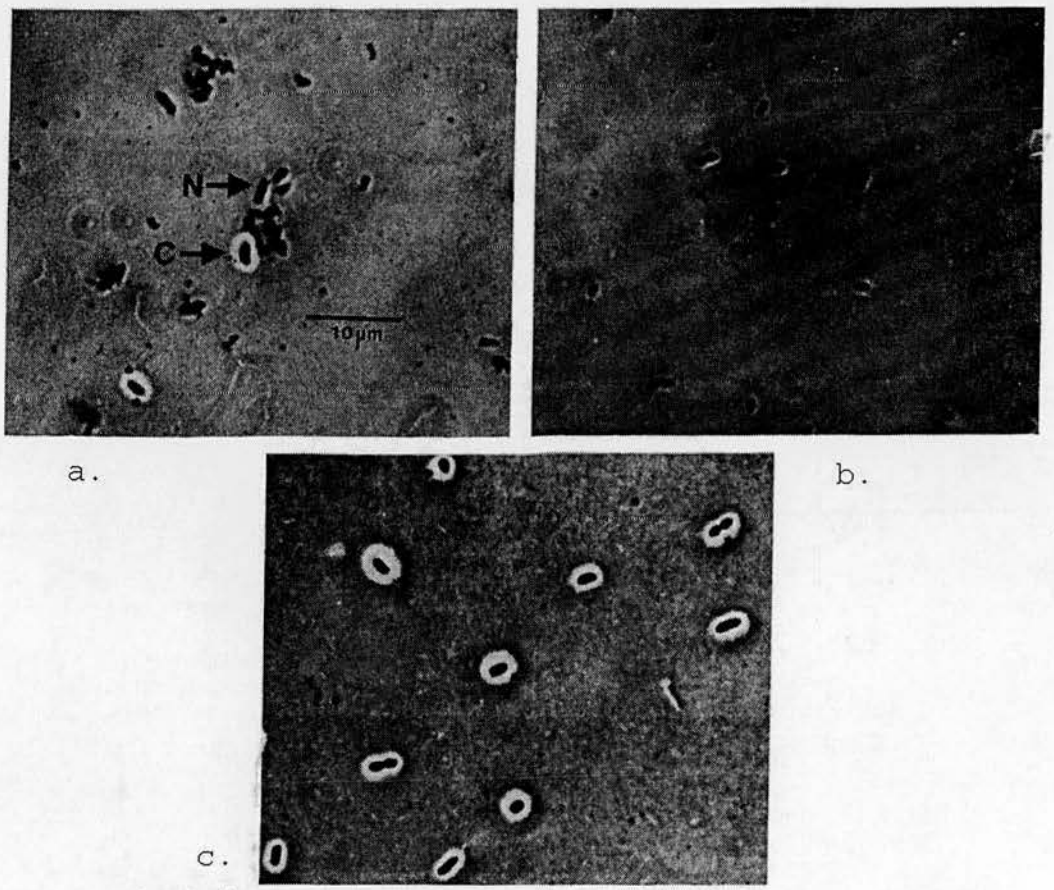

FIGURE. Photomicrographs of *Bacteroides fragilis* ATCC23745 grown in defined medium: a, culture before density gradient centrifugation; b, cells harvested from top of Percoll gradient after centrifugation; c, cells harvested from interface between 60% and 80% Percoll layers after centrifugation; C = capsulate cell; N = non-capsulate cell.

large capsules remained on top of the 20% layer and non-capsulate cells were concentrated at the 60–80% interface (figure). Most of the cells of *B. fragilis* strains ATCC23745 and NCTC10584 were found at either the 0–20% or the 60–80% interface. However, *B. fragilis* NCTC9343 produced additional bands at the 20–40% and 40–60% interfaces. These bands were formed by bacteria bearing capsules of graded sizes.

In subcultures of bacteria from the top layer of the density gradient, 90% of the cells of *B. fragilis* NCTC10584 and 99% of the cells of *B. fragilis* strains ATCC23745 and NCTC9343 were capsulate. Further subcultures of *B. fragilis* ATCC23745 were examined. Capsulate bacteria represented 95–100% of the total number of cells for the first three subcultures, after which the proportion of capsulate cells gradually diminished. Subculture of the 'non-capsulate' fractions resulted in an immediate return to the initial distribution of capsulate and non-capsulate cells.

Discussion

The method described produced homogeneous suspensions of cells with and without capsules. The capsulate fraction of *B. fragilis* strains ATCC23745 and NCTC9343 remained capsulate after subculture into defined broth and could be used in further studies. A proportion of cells of the non-capsulate fraction produced capsules when subcultured. This instability restricts the use of the non-capsulate fraction to investigations in aerobic conditions. In these conditions growth and biosynthesis of the capsule are prevented although viability of the phenotypically non-capsulate population is maintained. This useful technique has allowed us to proceed with in-vitro studies on the interaction of capsulate and non-capsulate *B. fragilis* with granulocytes and serum.

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Erratum

Correction to article by Patrick, S. and Reid, J.H. *J. med. Microbiol.*—vol. 16 (1983) 239–241.

p. 240. Figs. b and c were transposed; the caption should now read:

FIGURE . . . . . ; b, cells harvested from interface between 60% and 80% Percoll layers after centrifugation; c, cells harvested from top of Percoll gradient after centrifugation; . . . . . .

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THE GROWTH AND SURVIVAL OF CAPSULATE AND NON-CAPSULATE *BACTEROIDES FRAGILIS IN VIVO* AND *IN VITRO*

SHEILA PATRICK, J. H. REID AND M. J. LARKIN*

*Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast, BT12 6BN and * Sub-Department of Microbiology, David Keir Building, Stranmillis Road, Belfast, BT7 1NN*

SUMMARY. The growth of capsulate and non-capsulate *Bacteroides fragilis* in chambers implanted in the mouse peritoneal cavity was compared. Capsulate and essentially non-capsulate (< 1% capsulate) populations of *B. fragilis* strains NCTC9343 and NCTC10584 consistently grew exponentially to $> 10^9$ cfu/ml within 24 h *in vivo*, and low numbers of capsulate bacteria were maintained in the essentially non-capsulate population; however, the degree of capsulation of the capsulate population decreased by more than 60%. *B. fragilis* ATCC23745 differed from strains NCTC9343 and NCTC10584 in that growth was unpredictable and only occurred in some of the implanted chambers. Capsule production by cells of strain ATCC23745 varied from chamber to chamber: sometimes the proportion of capsulate cells increased after prolonged implantation. This could occur with either an increase or decrease in viable numbers *in vivo* and also after in-vitro incubation of this strain in chambers. The survival of capsulate and non-capsulate *B. fragilis* strains NCTC9343 and ATCC23745 was compared in aerobic and anaerobic conditions *in vitro*. In anaerobic conditions, capsulate and non-capsulate strain NCTC9343 survived equally well, whereas capsulate ATCC23745 survived better than its non-capsulate variants. Capsulate populations of both strains survived better than non-capsulate in aerobic conditions.

INTRODUCTION

In-vitro studies indicate that the capsule of *Bacteroides fragilis* impairs phagocytosis; however, its role in resistance to serum killing is less clear (Reid and Patrick, 1984). Smith (1980) stressed the importance of relating in-vitro investigations to growth *in vivo*. Animal models of intra-abdominal bacteroides infections have been reported by several authors. A study with rats indicated that *B. fragilis* ATCC23745 induced intra-abdominal abscess formation to a greater extent than other *Bacteroides* spp. (e.g., *B. distasonis*). This was attributed to the polysaccharide capsule of *B. fragilis*, because extracted polysaccharide alone caused abscesses. However, the

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inocula included barium sulphate and sterilised rat caecal contents, and the bacteria were suspended in glucose broth with peptone and yeast extract (PYG) (Onderdonk *et al.*, 1977). Kasper *et al.* (1980) used this model to obtain in-vivo passaged *B. fragilis* ATCC23745. O'Keefe *et al.* (1978) implanted punctured table-tennis balls into the rabbit peritoneal cavity. Three weeks later, a suspension of *B. fragilis* ATCC23745 in chopped-meat-glucose broth was inoculated into the table-tennis balls to provide a model for studies of the penetration and activity of penicillin G. McConville *et al.* (1981) challenged mice intraperitoneally with suspensions of sterile mouse faeces and a clinical isolate of *B. fragilis*. Sterile mouse faeces alone induced abscess formation, but more abscesses were produced when viable or heat-killed bacteria were added. PYG and sterilised mouse caecal contents are highly chemotactic for neutrophils in the absence of serum (Joiner *et al.*, 1980).

In this paper we report the growth of capsulate and essentially non-capsulate populations of *B. fragilis in vivo* and compare the survival of capsulate and non-capsulate bacteria *in vitro*. As it is difficult to determine the importance of the additives included in some animal models, we used a modification of the method of Day *et al.* (1980), in which bacteria are suspended in a salt solution and enclosed in a membrane-filter bound chamber. This initially allows free passage of diffusible host and bacterial factors, but excludes phagocytes.

Materials and methods

Bacterial strains. *B. fragilis* NCTC9343 was supplied by the Department of Bacteriology, University of Edinburgh Medical School, and *B. fragilis* ATCC23745 by the American Type Culture Collection, Rockville, MD. *B. fragilis* NCTC10584 was a departmental stock culture. Bacteria grown from these original cultures are referred to as normal (N) populations. N populations of strains NCTC9343 and NCTC10584 are $<1\%$ capsulate, whereas strain ATCC23745 is *c.* 17% capsulate (Patrick and Reid, 1983). Bacteria recovered from the 0–20% interface of a Percoll gradient are referred to as capsulate (C) and from the 60–80% interface as non-capsulate (NC).

Animals. Female C57 mice aged 8–12 weeks (given free access to food and water) were used in all experiments.

Bacterial culture methods. Cultures were incubated at 37°C in an atmosphere of 90% H_2 and 10% CO_2 in anaerobic jars. The standard anaerobic procedures of Collee *et al.* (1972) were used.

Stock cultures, grown in defined broth (Van Tassell and Wilkins, 1978), were snap-frozen in liquid N_2 and 1-ml portions were stored at -70°C or in liquid N_2. Capsulate bacteria, recovered from the 0–20% interface of a Percoll discontinuous density gradient (Patrick and Reid, 1983) and grown to late log-phase in defined broth, provided a C stock. This was stored as above.

Total viable counts were determined by seeding lysed human blood agar (LHBA) with six 20-μl drops from a standard 20 G 2-in steel cannula (Astell, London).

Preparation of inocula. Bacteria were grown to late log-phase in the defined broth. C cultures were then layered on to a cushion of 20% Percoll (Pharmacia, Fine Chemicals Inc., London), centrifuged at 2600 *g* for 20 min in a bench centrifuge, and the 100% C (supernatant) suspension removed. Non-capsulate (NC) suspensions were obtained from the 60–80% interface of a Percoll discontinuous density gradient.

Standard suspensions containing $(2–5)\times10^7$ cfu/ml were prepared in quarter-strength Ringer solution (Oxoid) with 0·05% free base of cysteine (RS+cys) and checked with a Pye Unicam SP 800 spectrophotometer.

In-vitro growth. Triplicate tubes of defined broth were inoculated for each sampling time at a concentration of $(2–5)\times10^7$ cfu/ml. Each set of replicate tubes was incubated in a separate anaerobic jar and only sampled once.

In-vivo growth. Day *et al.* (1980) described a model for in-vivo growth of bacteria in chambers implanted intraperitoneally in mice. We used this method when chambers containing strain ATCC23745 were prepared in an anaerobic cabinet (Forma Scientific Inc.; gas mixture 85% N_2, 10% H_2, 5% CO_2). Because the method leaves a gas space inside the chamber, the following modification was used in all other experiments when chambers were prepared in aerobic conditions.

Chambers were made from 10-mm lengths of 1-ml polypropylene syringe barrels (Becton and Dickinson, Dublin). Two holes were made in the sides of the chambers, towards each end, with a red-hot 25 G needle. A membrane filter (0·45-μm pore; Millipore, Harrow, Middlesex) was stuck aseptically on to each end of the barrel with UHU glue (Beecham UHU, Brentford, Middlesex) and the glue allowed to harden. The chambers were filled through the sides with standard bacterial suspensions. A 2-ml syringe with a 26 G needle was inserted into one of the holes and the chamber filled until all the air was excluded (final volume *c.* 0·2 ml). The two holes were then sealed with UHU glue and the chambers transferred into bijou bottles containing *c.* 4·5 ml of RS + cys. These were then stored in an anaerobic jar with an activated Gas Generating Kit (Oxoid) at room temperature until implanted into the peritoneal cavity of mice; (two chambers were inplanted in each mouse). LHBA plates were spread with 1 ml of the RS + cys from the bijou bottles to check for leaking chambers.

Duplicate mice were killed after the required incubation period and the chambers removed. The membrane filters were punctured with a syringe needle and the chambers washed four times with 0·2 ml RS. The numbers of viable bacteria in each chamber were determined.

The degree of bacterial capsulation was determined directly by examination of the suspensions obtained from the chambers, and indirectly by examination of the late log-phase growth after one subculture in defined broth. Standard suspensions of < 1% capsulate (N) *B. fragilis* strains NCTC9343 and NCTC10584, non-capsulate *B. fragilis* ATCC 23745 and capsulate populations of all three strains were used as inocula.

Bacterial survival in vitro. Five-ml volumes of standard suspensions in RS + cys of C and NC variants of *B. fragilis* NCTC9343 and ATCC23745 were incubated in bijou bottles aerobically and anaerobically at 37°C for 24 h. The initial and final viable counts were then compared. Survival of strain ATCC23745 in anaerobically-prepared chambers placed in bijou bottles containing RS + cys was also determined. Degree of capsulation was determined in a late log-phase subculture in defined broth.

Microscopy. Capsulation was determined by light microscopy with eosin-carbol fuchsin negative staining (Cruickshank, 1965) and by electronmicroscopy wth ruthenium red staining (Springer and Roth, 1973).

The presence of phagocytes on the chamber membrane filters was determined by light microscopy of methanol-fixed filters stained with M.D.Diff-Quick solutions 1 and 2 (Merz and Dade AG, Switzerland).

Statistical analyses. Growth curves of capsulate and normal populations were compared by a two-way analysis of variance with linear regression. Growth rates were compared by a test of significance between the regressions and the length of the lag-phase by comparison of the blocks of data (F tests) with a Texas T1 59 programmable calculator (Parker, 1979). The survival of capsulate and non-capsulate bacteria *in vitro* was compared by Student's 't' test. The bars included in the figures represent the standard errors.

RESULTS

Microscopy of bacteria grown in chambers in-vivo

Suspensions of bacteria obtained from the chambers were examined directly with eosin-carbol fuchsin staining. Bacteria grown *in vivo* were smaller than those grown in defined broth: for example, cells of strain NCTC9343 grown *in vivo* were *c.* 0·7 × 1·0–3·6 μm whereas cells grown in defined broth were 0·9 × 1·4–5·1 μm. It was, therefore, difficult to determine the proportion of capsulate cells accurately because the

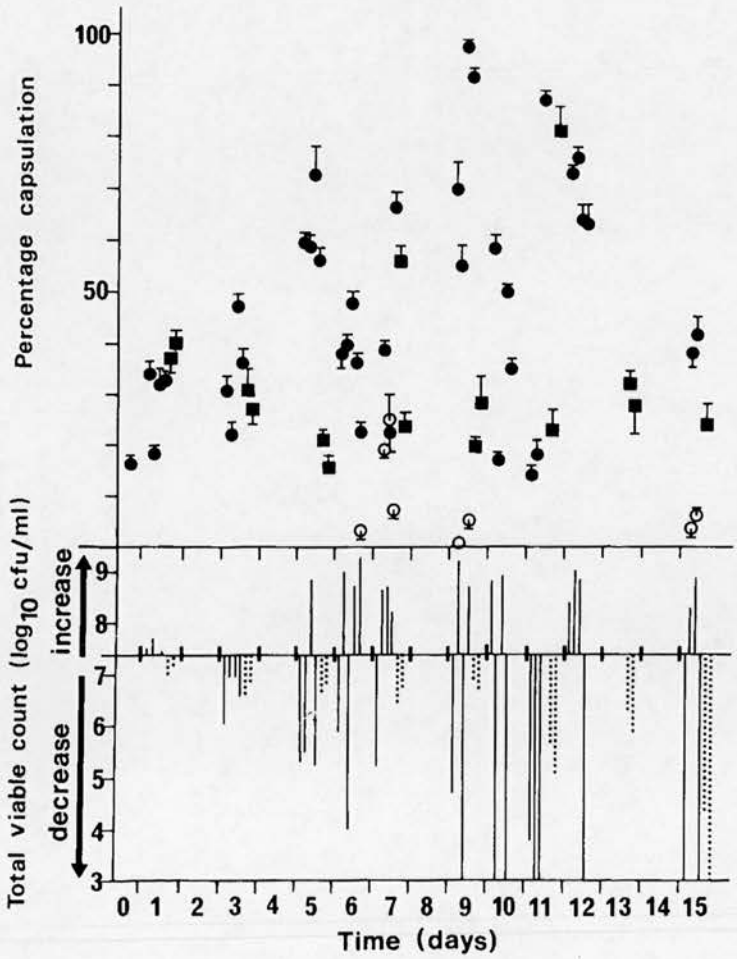

FIG. 1.—Survival, growth and percentage capsulation of initially *c.* 17% capsulate *B. fragilis* ATCC23745 *in vivo* and *in vitro* in chambers constructed in anaerobic conditions: increase or decrease in total viable count (log_{10} cfu/ml) *in vivo* (I) and *in vitro* (⋮); percentage capsulation determined directly on in-vivo grown bacteria (○) and, after one in-vitro subculture of bacteria incubated *in vivo* (●) and *in vitro* (■). Each symbol represents the results from one chamber. ⌶ = standard error in all figs.

eosin-carbol fuchsin stain sometimes shrinks to leave a bright halo. Bacteria grown *in vivo* were subcultured once in defined broth and the proportions of capsulate cells were compared with direct determinations on chamber suspensions.

Strains NCTC9343 and NCTC10584 grown *in vivo* showed an apparent decrease in the proportion of capsulate cells after only one subculture *in vitro*; this was quite reproducible in replicate experiments. However, the percentage capsulation of *B. fragilis* ATCC23745 grown *in vivo* increased to a varying extent on in-vitro subculture (fig. 1). Some of these bacteria may not produce capsules *in vivo*, while retaining the potential for capsule production, or the capsules may be too small to be seen with the light microscope. Ruthenium red staining and electronmicroscopy showed the presence of a thick dark-staining layer outside the membrane on some of the cells. This resembled the ruthenium red-staining capsule desribed by Kasper (1976).

Because of these anomalies, the proportion of cells grown *in vivo* capable of producing capsules after one in-vitro subculture was used as an indicator of capsulation for all three strains.

Observation of the implanted chambers

Filters from the ends of the chambers were stained by the Diff-Quick method and examined microscopically. There was a gradual build up of leukocytes on the outer

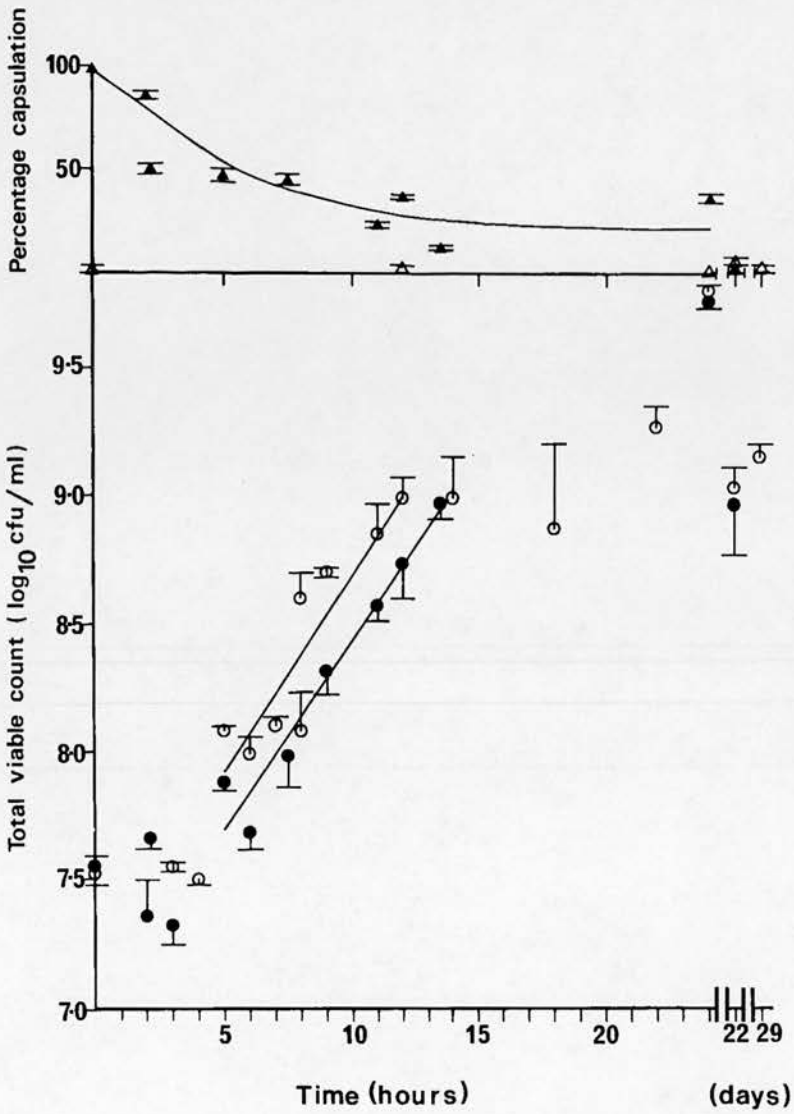

FIG. 2.—In-vivo growth curve of initially 100% capsulate (●) and < 1% capsulate (○) *B. fragilis* NCTC9343 and percentage capsulation of bacteria grown *in vivo* after one in-vitro subculture, initially 100% capsulate (▲) and < 1% capsulate (△). The results from five separate experiments are combined for each growth curve and each point represents the mean of four chambers. Specific growth rate of 100% $C = 0{\cdot}34 \pm 0{\cdot}09$/h and of < 1% $C = 0{\cdot}35 \pm 0{\cdot}18$/h.

surfaces of the filters on chambers containing C and N populations. Leukocytes were not observed on the inner surfaces of the filters. After prolonged incubation, the chambers in which growth occurred became encased in fibrin and granulation tissue, as described by Day *et al.* (1980) in their work with chambers containing *Staphylococcus aureus*.

Growth and survival of B. fragilis

During incubation *in vivo* for 24 h, C and N populations of *B. fragilis* NCTC9343 grew exponentially to $> 10^9$ cfu/ml and maintained high viable numbers for at least 22 days (fig. 2). Throughout this period the proportion of capsulate cells in the N population remained low. However, the proportion of capsulate cells in the C population steadily decreased during 22 days to that of the N population. In contrast,

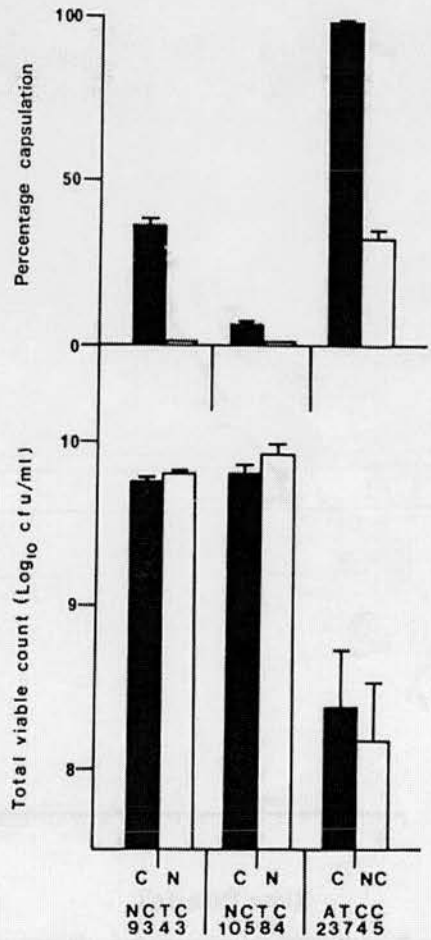

FIG. 3.—Comparison of total viable count and percentage capsulation (after one in-vitro subculture) of initially 100% capsulate (C) and < 1% capsulate (N) *B. fragilis* strains NCTC9343 and NCTC10584 and, initially C and non-capsulate (NC) strain ATCC23745. Each bar represents the mean of four chambers and the results are representative of more than one experiment.

C populations subcultured in defined broth maintained a high level of capsulation (Patrick and Reid, 1983). The C population had a significantly longer lag-phase than the N population *in vivo*, although the growth rates did not differ significantly. Growth *in vivo* was slower than in defined broth, with generation times for C bacteria of 2·06 h *in vivo* and 1·60 h in defined broth, and for N bacteria of 1·96 h *in vivo* and 1·42 h in defined broth. The difference in the lag-phase and the selection against capsulate cells *in vivo* was not attributable to poor survival in RS + cys. When incubated *in vitro* at 37°C for 24 h, C and N populations of *B. fragilis* NCTC9343 maintained similar viable numbers anaerobically, whereas the capsulate population survived better than the non-capsulate in aerobic conditions. *B. fragilis* NCTC10584 behaved similarly, but strain ATCC23745 did not (fig. 3).

B. fragilis ATCC23745 did not grow exponentially during incubation for 24 h *in vivo* (fig. 3), even if the chambers were constructed and filled in an anaerobic cabinet (fig. 1). Growth did occur in some chambers after prolonged incubation, but it was erratic. In-vivo incubation tended to increase the proportion of cells capable of producing capsules, whether growth occurred or not, but again this was inconsistent. The percentage capsulation also increased in some control chambers incubated *in vitro*

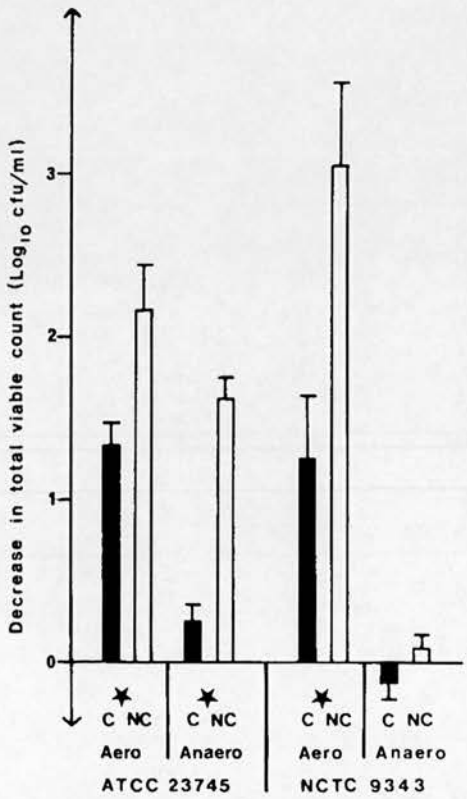

FIG. 4.—Survival of 100% capsulate (C) and non-capsulate (NC) *B. fragilis* strains NCTC9343 and ATCC23745 incubated aerobically (Aero) and anaerobically (Anaero) in Ringer solution + free base of cysteine (0·05%) at 37°C for 24 h. Each bar represents the combined results from two separate experiments. ✱ = difference between C and NC statistically significant ($p = 0·05$).

in RS + cys at 37°C (fig. 1). The results summarised in fig. 4 show that the capsule of ATCC23745 enhances survival aerobically and anaerobically; this could be relevant to the selection of a capsulate population.

Discussion

In this model, *B. fragilis* grew in chambers implanted intraperitoneally in mice in the absence of phagocytes and without the addition of adjuvants such as barium sulphate, sterile mouse faeces or growth medium. However, the physical presence of the chamber may enhance bacterial growth as the presence of a foreign body in the form of a subcutaneously implanted polytetrafluoroethylene tube in guinea pigs enhanced the growth of *S. aureus* (Zimmerli *et al.*, 1982).

The present study shows that, in the absence of phagocytes, non-capsulate *B. fragilis* strains NCTC9343 and NCTC10584 can proliferate *in vivo* and have a selective advantage over capsulate organisms. The reasons for this are not known, but do not relate to survival aerobically or anaerobically *in vitro* in Ringer solution with cysteine. Any differences in the growth rate of the capsulate and non-capsulate variants will influence the proportions of these cell types in the population, as would the frequency of switching from one state to the other. The relative importance of these two factors remains to be determined. Rapid phase or form variation in surface structures of pathogenic bacteria, without loss of the potential for production, could be an advantage in a changing host environment (Mäkelä *et al.*, 1980). In studies of *B. thetaiotaomicron* by Burt *et al.* (1978), capsulate cells changed to non-capsulate at a frequency of $1{\cdot}1 \times 10^{-2}$ and non-capsulate to capsulate at $1{\cdot}4 \times 10^{-2}$ *in vitro* at 37°C. Capsulation is related to colony form in *B. thetaiotaomicron*, but no change in colony morphology of capsulate and non-capsulate *B. fragilis* has been observed in the present study.

B. fragilis ATCC23745 differed from the other two strains in that growth *in vivo* in implanted chambers was unpredictable. There was also a trend towards a higher proportion of capsulate cells with longer incubation, whether in-vivo growth occurred or not. O'Keefe *et al.* (1978) also reported a wide variation in viable counts of *B. fragilis* ATCC23745 *in vivo*. Table-tennis balls implanted intraperitoneally (IP) into rabbits were inoculated with 1 ml of broth culture containing 10^9–10^{10} cfu/ml. After incubation for 4 or 5 days the same dose of penicillin G was given to all the rabbits, and viable counts then ranged from 10^3–10^{10} cfu/ml. This variation probably occurred during the 4–5-day incubation period and was not attributable to antibiotic treatment, because penicillin G was biologically inactive in heavily infected ($> 10^9$ cfu/ml) balls. *B. fragilis* ATCC23745 isolated from experimental intraperitoneal abscesses in rats (Kasper *et al.*, 1980) and subcutaneous abscesses in mice (Simon *et al.*, 1982) produced more capsular material than the same strain serially subcultured only on blood agar. *B. fragilis* ATCC23745, isolated from experimental subcutaneous abscesses in mice, maintained higher viable numbers in the rabbit intraperitoneal table-tennis ball model of O'Keefe *et al.* (1978) than *B. fragilis* ATCC23745 which had been subcultured ten times on blood agar (Simon *et al.*, 1982). The present study shows that the degree of capsulation of strain ATCC23745 can also be enhanced without incubation *in vivo* and that the capsule enhances in-vitro survival aerobically and anaerobically.

Although the reasons for the different behaviour of strains NCTC9343 and

NCTC10584, and ATCC23745 are not clear, the following facts should perhaps be given further consideration. Strain ATCC23745 was isolated from pleural fluid and was initially named *Sphaerophorus intermedius* (Bergan and Hovig, 1968), whereas strain NCTC9343 was isolated from an appendix abscess and NCTC10584 from pus. The polysaccharide capsules of strains ATCC23745 and NCTC9343 have been shown to be chemically and immunochemically different, although the lipopolysaccharide is similar (Kasper *et al.*, 1983). In-vitro cultures of strain ATCC23745 have a higher proportion of capsulate cells than strains NCTC9343 and NCTC10584 (Patrick and Reid, 1983). Spherical bodies with multilayered walls, sometimes associated with bacteriophage, have been observed in *B. fragilis* strain ATCC23745 but not in other strains examined (Silver *et al.*, 1975; Reid, 1981). Booth *et al.* (1979) reported a phage-carrier state in strains of *B. fragilis* that produced thick capsules. When these cultures were cured of phage the proportion of capsulate cells decreased. The activity of bacteriophage in strain ATCC23745 could be a factor involved in the differences observed in the present study, but direct evidence for this has yet to be obtained.

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PHAGOCYTIC AND SERUM KILLING OF CAPSULATE AND NON-CAPSULATE *BACTEROIDES FRAGILIS*

J. H. REID AND SHEILA PATRICK

Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast, BT12 6BN, Northern Ireland

SUMMARY. The relative susceptibilities of capsulate and non-capsulate variants of *Bacteroides fragilis* to serum and phagocytic killing were investigated. The capsule of *B. fragilis* did not confer resistance to serum killing. Phagocytic killing of non-capsulate *B. fragilis* occurred at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml. Capsulate *B. fragilis* organisms were also phagocytosed and killed at a concentration of 1×10^6 cfu/ml, but phagocytosis and killing were impaired at a concentration of 1×10^7 cfu/ml.

INTRODUCTION

The susceptibility of pathogenic bacteria to phagocytosis and killing by polymorphonuclear leukocytes (PMNL) and macrophages is of major importance in determining the outcome of the host-pathogen interaction. Casciato *et al.* (1975) and Bjornson, Altemeier and Bjornson (1976) demonstrated phagocytosis and killing of *B. fragilis* by human leukocytes *in vitro*. Phagocytosis of *B. fragilis* in the presence of serum occurred in aerobic and anaerobic conditions. Ingham *et al.* (1977 and 1981) investigated the effect of *Bacteroides* spp. on the phagocytic killing of facultative species. Killing of *B. fragilis* and *Proteus mirabilis* in mixtures *in vitro* was impaired when the concentration of *B. fragilis* was greater than 1×10^7 cfu/ml in the phagocytic system. Tofte *et al.* (1980) and Jones and Gemmel (1982) reported that both phagocytic uptake and killing of facultative species were impaired at high concentrations of bacteroides. None of these studies defined the ratio of capsulate to non-capsulate bacteroides used in the phagocytic system.

Considerable emphasis has been placed on the importance of the polysaccharide capsule of *B. fragilis* ATCC23745 as a virulence determinant (Kasper *et al.*, 1977; Onderdonk *et al.*, 1977). Kasper *et al.* (1980) reported that in-vivo passage of this strain enhanced the production of the capsule but serial subculture *in vitro* reduced the proportion of capsulate bacteria. Simon *et al.* (1982) compared phagocytosis of these strains and successfully demonstrated a reduction in uptake of the in-vivo passaged variant.

Recently, Percoll density gradient centrifugation was successfully used to separate capsulate and non-capsulate variants of *B. fragilis* (Patrick and Reid, 1983). In the

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present study, the susceptibilities of these variants to phagocytosis, intracellular killing and the bactericidal action of serum were investigated.

Materials and methods

Bacterial strains. *B. fragilis* NCTC9343 was supplied by the Department of Bacteriology, University of Edinburgh Medical School; *B. fragilis* ATCC23745 by the American Type Culture Collection, Rockville, MD; and *B. fragilis* NCTC10584 was a departmental stock culture.

Bacterial culture methods. Bacteria grown in defined broth (Van Tassell and Wilkins, 1978) were used in all experiments except where otherwise stated. Cultures were incubated at 37°C in an atmosphere of H_2 90% and CO_2 10% in anaerobic jars. The standard anaerobic procedures of Collee *et al.* (1972) were used.

Stock cultures, grown in defined broth, were snap frozen in liquid N_2 and 1-ml portions stored at −70°C or in liquid N_2. Capsulate bacteria, recovered from the 0–20% interface of a Percoll (Pharmacia, London) discontinuous density gradient (Patrick and Reid, 1983) and grown to late log-phase in defined broth, provided a capsulate stock which was stored as above.

Bacteria were also cultured in basal broth (Deacon, Duerden and Holbrook, 1978). Total viable counts were determined by seeding lysed human blood agar with six 20-μl drops from a standard 20-gauge steel cannula (Astell, London).

Preparation of bacteria. Bacteria were grown to late log-phase in defined broth medium. A 3-ml volume of a capsulate culture preparation was layered on to a 20% Percoll cushion. After centrifugation at 2600 *g* for 20 min a homogeneous suspension of capsulate bacteria was recovered from the 0–20% interface, suspended in quarter strength Ringer solution (Oxoid, Basingstoke, Hampshire) and centrifuged at 10 000 *g* for 30 min at 4°C. Non-capsulate bacteria obtained from the 60–80% interface of a Percoll density gradient were also washed once in Ringer solution. Standard bacterial suspensions at concentrations of 1×10^7 or 1×10^8 cfu/ml were prepared in Hanks' balanced salt solution (HBSS) containing gelatin 0·01% w/v (GEL-HBSS).

Preparation of phagocytes. Ficoll isopaque gradients were used to separate leukocytes from 25 ml of heparinised human blood. Leukocytes were washed twice in HBSS, and pure PMNL preparations were obtained by centrifugation of the leukocyte suspension on a preformed Percoll sucrose density gradient.

A stock solution of Percoll, isosmotic with physiological saline, was prepared by diluting nine volumes of Percoll in one volume of tenfold concentrated HBSS. The *p*H of this solution was adjusted to 7·2 with 1M HCl, and a 65% Percoll solution was then prepared by the addition of 0·25 M sucrose. Suitable volumes were centrifuged at 21 000 *g* for 20 min to produce gradients. Leukocytes suspended in 1 ml of HBSS were applied to the top of a gradient which was then centrifuged at 600 *g* for 15 min. A sharp band of PMNL formed in the lower part of the gradient just above the band of erythrocytes; the cells were removed carefully with a Pasteur pipette, washed in HBSS and resuspended to a final concentration of 1×10^7 cells/ml.

Serum. Normal human group AB serum was obtained from the Northern Ireland Blood Transfusion Service; 2-ml volumes of sera were stored at −20°C. The total haemolytic complement value (CH50) was checked routinely, and only sera with normal CH50 values were used.

Phagocytosis. Sterile siliconised screw-capped glass tubes (98 × 16 mm) containing a phagocytic system composed of 0·3 ml of PMNL suspension, 0·3 ml of AB serum, 0·3 ml of appropriate bacterial suspension and 2·1 ml of GEL-HBSS were incubated at 37°C in aerobic conditions with end-over-end rotation. GEL-HBSS was used to bring the final volume to 3·0 ml. Final PMNL and serum concentrations were 1×10^6 cells/ml and 10% respectively, and final bacterial concentrations were either 1×10^7 or 1×10^6 cfu/ml. Appropriate control mixtures were included. All experiments were performed in duplicate and repeated at least twice. Phagocytic killing at 0 and 120 min was measured by diluting 0·1 ml of the reaction mixture into 9·9 ml of distilled water containing bovine serum albumin 0·01%. After 5 min, further tenfold dilutions were performed in Ringer solution and total viable counts were determined. Phagocytic uptake after 0, 30, 60 and 120 min was measured by diluting 0·5 ml of

the reaction mixture in 1·5 ml of chilled GEL-HBSS to stop phagocytosis. The suspension was centrifuged at 110 *g* for 4 min at 4°C and the viable bacteria in the supernate were counted.

Light microscopy. Capsules were detected by light microscopy with India ink or eosin-carbol fuchsin negative staining methods (Cruickshank, Duguid and Swain, 1965).

PMNL were recovered from the phagocytic system by centrifugation at 210 *g* for 4 min and resuspended in HBSS containing 50% heat-inactivated normal human serum. Smears were prepared and stained by the M & D Diff-Quick® staining set (Merz and Dade AG, Switzerland). Preparations were examined with a × 100 oil immersion objective and the number of bacteria/50 PMNL counted.

Electronmicroscopy. PMNL for electronmicroscopy were recovered from the phagocytic system by centrifugation at 210 *g* for 4 min, resuspended in 0·1 M Sorensen's phosphate buffer *p*H 7·3 (SPB) containing glutaraldehyde 2·5% v/v and left at 4°C for 1 h. Cells were washed twice in SPB, resuspended in SPB containing osmic acid 1% v/v, left at 22°C for 1h and then washed twice in SPB, dehydrated in graded ethyl alcohols and embedded in Spurr resin. Sections for electronmicroscopy were stained with uranyl acetate and lead citrate and viewed in a Philips 301 transmission electronmicroscope.

RESULTS

Bactericidal activity of human serum

Capsulate and non-capsulate variants of two of the three test strains (NCTC9343 and NCTC10584) showed no reduction in viable counts after incubation for 2 h in 10% normal human serum (table I). The non-capsulate variant of *B. fragilis* ATCC23745 was also resistant to serum killing, but the capsulate variant of this strain was susceptible to the bactericidal action of 10% normal human serum (table I). Control experiments indicated that GEL-HBSS was not toxic to capsulate *B. fragilis* ATCC23745.

TABLE I

Susceptibility of capsulate and non-capsulate variants of B. fragilis to serum and phagocytic killing

Organism	Ratio of bacteria to PMNL	Total viable count* (cfu/ml) after 0 min	Total viable count* (cfu/ml) after 120 min
B. fragilis NCTC9343 (NC)	10:1	$1{\cdot}5 \pm 0{\cdot}2 \times 10^7$	$3{\cdot}5 \pm 2{\cdot}8 \times 10^6$
	1:1	$1{\cdot}4 \pm 0{\cdot}2 \times 10^6$	$1{\cdot}0 \pm 0{\cdot}1 \times 10^5$
	Serum control	$1{\cdot}3 \pm 0{\cdot}1 \times 10^6$	$1{\cdot}5 \pm 0{\cdot}2 \times 10^6$
B. fragilis NCTC9343 (C)	10:1	$1{\cdot}1 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}1 \pm 0{\cdot}1 \times 10^7$
	1:1	$1{\cdot}1 \pm 0{\cdot}1 \times 10^6$	$5{\cdot}2 \pm 0{\cdot}9 \times 10^4$
	Serum control	$1{\cdot}2 \pm 0{\cdot}1 \times 10^6$	$1{\cdot}0 \pm 0{\cdot}1 \times 10^6$
B. fragilis NCTC10584 (NC)	10:1	$1{\cdot}7 \pm 0{\cdot}2 \times 10^7$	$4{\cdot}6 \pm 0{\cdot}9 \times 10^6$
	1:1	$2{\cdot}1 \pm 0{\cdot}1 \times 10^6$	$0{\cdot}9 \pm 0{\cdot}1 \times 10^5$
	Serum control	$1{\cdot}9 \pm 0{\cdot}1 \times 10^6$	$2{\cdot}1 \pm 0{\cdot}2 \times 10^6$
B. fragilis NCTC10584(C)	10:1	$1{\cdot}9 \pm 0{\cdot}2 \times 10^7$	$1{\cdot}8 \pm 0{\cdot}3 \times 10^7$
	1:1	$1{\cdot}9 \pm 0{\cdot}1 \times 10^6$	$5{\cdot}5 \pm 0{\cdot}3 \times 10^5$
	Serum control	$1{\cdot}8 \pm 0{\cdot}1 \times 10^6$	$1{\cdot}9 \pm 0{\cdot}2 \times 10^6$
B. fragilis ATCC23745 (NC)	10:1	$2{\cdot}9 \pm 0{\cdot}3 \times 10^7$	$3{\cdot}0 \pm 0{\cdot}2 \times 10^6$
	1:1	$2{\cdot}2 \pm 0{\cdot}4 \times 10^6$	$3{\cdot}5 \pm 1{\cdot}0 \times 10^4$
	Serum control	$2{\cdot}3 \pm 0{\cdot}2 \times 10^6$	$2{\cdot}5 \pm 0{\cdot}2 \times 10^6$
B. fragilis ATCC23745 (C)	10:1	$0{\cdot}7 \pm 0{\cdot}1 \times 10^7$	$1{\cdot}0 \pm 0{\cdot}9 \times 10^5$
	1:1	$1{\cdot}2 \pm 0{\cdot}1 \times 10^6$	$1{\cdot}5 \pm 0{\cdot}4 \times 10^4$
	Serum control	$1{\cdot}1 \pm 0{\cdot}1 \times 10^6$	$2{\cdot}2 \pm 0{\cdot}5 \times 10^4$

PMNL = polymorphonuclear leukocyte; C = capsulate; NC = non-capsulate.
* = mean ± SE.

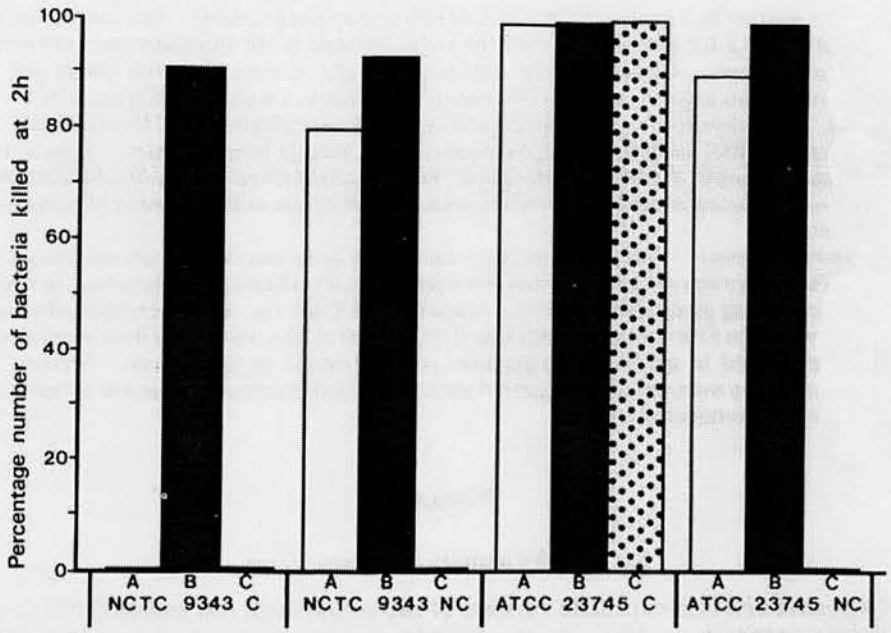

FIG. 1.—A comparison of killing of capsulate (C) and non-capsulate (NC) variants of *B. fragilis* NCTC9343 and ATCC23745 by PMNL and serum. A—bacteria to PMNL ratio = 10:1; B—bacteria to PMNL ratio = 1:1; C—bacteria without PMNL; all mixtures contained serum.

Killing by polymorphonuclear leukocytes

The results in table I and fig. 1 indicate that phagocytic killing of the three non-capsulate strains of *B. fragilis* grown in defined broth occurred at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml (i.e., bacteria to PMNL ratios of 1:1 and 10:1 respectively). Non-capsulate *B. fragilis* NCTC9343 grown in basal broth was also susceptible to phagocytic killing at both bacterial concentrations (table II).

The capsulate variants of *B. fragilis* NCTC9343 and NCTC10584 were killed by

TABLE II

Susceptibility of capsulate and non-capsulate variants of B. fragilis NCTC9343 grown in basal broth to phagocytic killing by PMNL

State of test organism	Ratio of bacteria to PMNL	Total viable count* (cfu/ml) after 0 min	Total viable count* (cfu/ml) after 120 min	Percentage number of bacteria killed
Capsulate	10:1	$3{\cdot}2 \pm 0{\cdot}2 \times 10^7$	$9{\cdot}1 \pm 0{\cdot}5 \times 10^6$	72
	1:1	$6{\cdot}1 \pm 0{\cdot}4 \times 10^6$	$3{\cdot}6 \pm 0{\cdot}2 \times 10^5$	92
	Serum control	$6{\cdot}7 \pm 0{\cdot}7 \times 10^6$	$7{\cdot}0 \pm 0{\cdot}5 \times 10^6$	0
Non-capsulate	10:1	$2{\cdot}9 \pm 0{\cdot}1 \times 10^7$	$7{\cdot}4 \pm 0{\cdot}3 \times 10^6$	75
	1:1	$8{\cdot}5 \pm 0{\cdot}5 \times 10^6$	$5{\cdot}2 \pm 0{\cdot}4 \times 10^5$	94
	Serum control	$6{\cdot}7 \pm 0{\cdot}2 \times 10^6$	$6{\cdot}6 \pm 0{\cdot}3 \times 10^6$	0

PMNL = Polymorphonuclear leukocyte.
* = Mean ± SE.

PMNL at bacterial concentrations of 1×10^6 cfu/ml. However, when the bacterial concentration was increased to 1×10^7 cfu/ml, phagocytic killing of these strains was inhibited (table I and fig. 1). A high proportion (>70%) of capsulate *B. fragilis* NCTC9343 grown in basal broth was susceptible to phagocytic killing at both bacterial concentrations (table II). Killing of capsulate *B. fragilis* ATCC23745 occurred at both bacterial concentrations; however, this strain was sensitive to the bactericidal action of normal human serum (table 1 and fig. 1).

Phagocytosis and killing by polymorphonuclear leukocytes

The rates of phagocytosis and killing of capsulate and non-capsulate variants of *B. fragilis* NCTC9343 were compared. At bacterial concentrations of 1×10^7 cfu/ml, effective phagocytosis and killing of non-capsulate *B. fragilis* NCTC9343 was evident

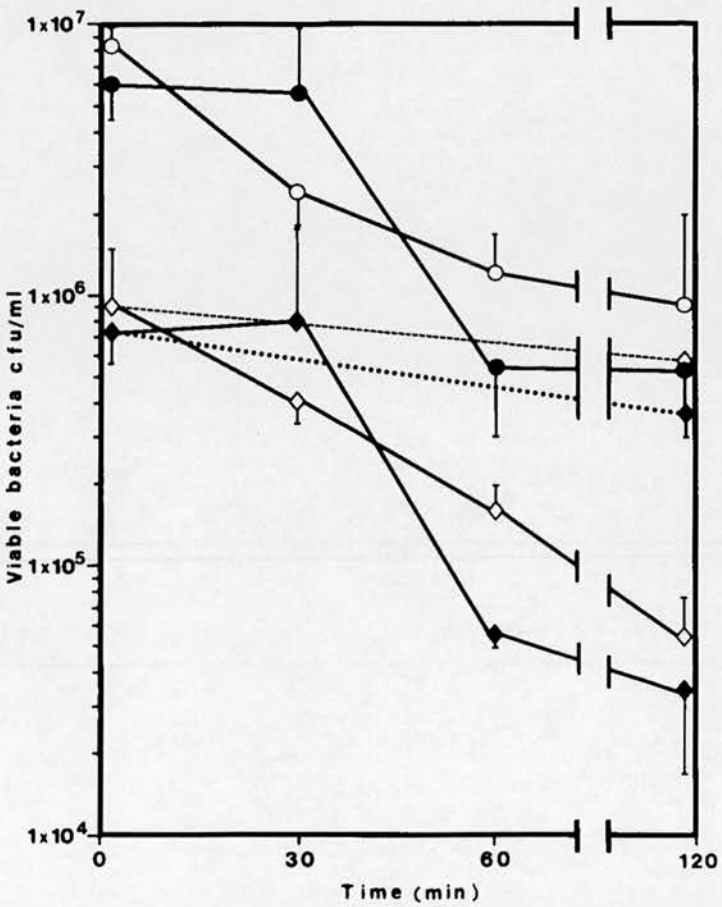

FIG. 2.—Phagocytosis and killing of non-capsulate *B. fragilis* NCTC9343 during incubation with 10% serum and PMNL. Phagocytic uptake (●) and killing (○) at a bacterial concentration of 1×10^7 cfu/ml; phagocytic uptake (♦) and killing (◇) at a bacterial concentration of 1×10^6 cfu/ml. Controls for uptake (. . . .) and killing (- - - -) were bacteria incubated in serum without PMNL. Results from experiments performed on 3 days were combined and expressed as mean ±SE.

after incubation for 60 min (fig. 2). However, phagocytosis of capsulate *B. fragilis* NCTC9343 was impaired after incubation for 60 min and no killing occurred (fig. 3). At bacterial concentrations of 1×10^6 cfu/ml, phagocytosis and killing were similar with each variant (figs 2 and 3).

Microscopy

Phagocyte-associated bacteria were difficult to detect at a low bacteria to PMNL ratio (1:1) by light microscopy with differential staining. Smears prepared from the phagocytic system containing non-capsulate *B. fragilis* NCTC9343 at a high bacteria to PMNL ratio (10:1) showed numerous PMNL-associated bacteria. When *B. fragilis* NCTC9343 was grown in defined broth the mean number of non-capsulate bacteria per PMNL was 11; growth in basal broth increased the number of

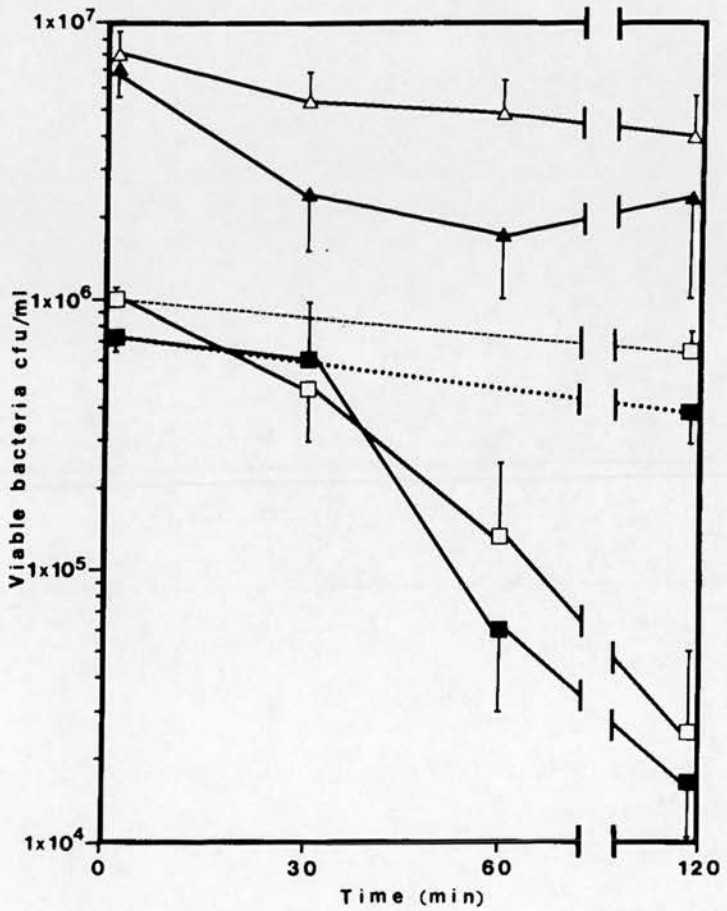

FIG. 3.—Phagocytosis and killing of capsulate *B. fragilis* NCTC9343 during incubation with 10% serum and PMNL. Phagocytic uptake (▲) and killing (△) at a bacterial concentration of 1×10^7 cfu/ml; phagocytic uptake (■) and killing (□) at a bacterial concentration of 1×10^6 cfu/ml. Controls for uptake (. . . .) and killing (- - - -) were bacteria incubated in serum without PMNL. Results from experiments performed on 3 days were combined and expressed as mean ±SE.

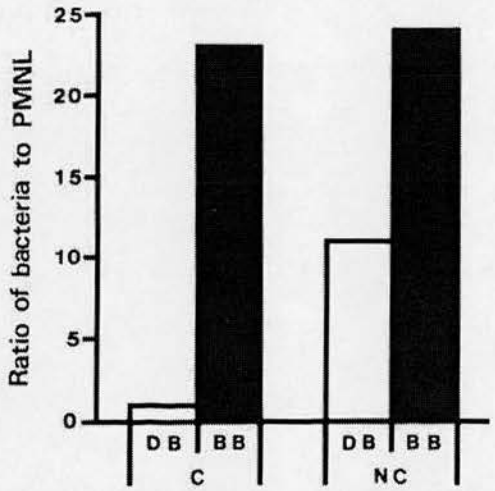

FIG. 4.—*B. fragilis* NCTC9343 associated with PMNL after incubation for 60 min in 10% serum at 37°C. DB = defined broth; BB = basal broth; C = capsulate; NC = non-capsulate.

phagocyte-associated bacteria to 24 (fig. 4). Electronmicroscopy confirmed the intracellular location of these organisms and demonstrated that bacteria were surrounded by phagosomal membranes (fig. 5A).

Few PMNL-associated capsulate *B. fragilis* NCTC9343 were observed at a bacteria to PMNL ratio of 10:1 (figs 4 and 5B). Electronmicroscopy revealed that some capsulate organisms were associated with the outside of the PMNL membrane (fig. 6). However, high numbers of capsulate *B. fragilis* NCTC9343 grown in basal broth were observed within phagocytes at a bacteria to PMNL ratio of 10:1 (fig. 4).

DISCUSSION

These findings indicate that the capsule of *B. fragilis* is not responsible for conferring resistance to serum killing.

Non-capsulate *B. fragilis* ATCC23745 grown in defined broth was resistant to serum killing, but the capsulate variant of this strain was susceptible to the bactericidal action of 10% normal human serum. The phagocytic system used in our study contained 10% normal human serum and viable counting methods were used to detect phagocytosis and killing. Phagocytosis of serum-sensitive strains could not be investigated because phagocytic killing could not be distinguished from serum killing.

Optimum phagocytic killing of both capsulate and non-capsulate *B. fragilis* occurred at bacterial concentrations of 1×10^6 cfu/ml, but differences in the susceptibility to phagocytic killing were observed at bacterial concentrations of 1×10^7 cfu/ml. The results of our uptake and electronmicroscopy experiments suggest that ingestion of capsulate *B. fragilis* was impaired at bacterial concentrations of 1×10^7 cfu/ml. The surface of a particle must be completely coated with opsonin for successful phagocytosis (Griffin *et al.*, 1975). Incomplete opsonisation of capsulate *B. fragilis* at high bacterial concentrations with limited amounts of an essential opsonin (e.g., IgM) could result in the impaired phagocytosis observed in our experiments.

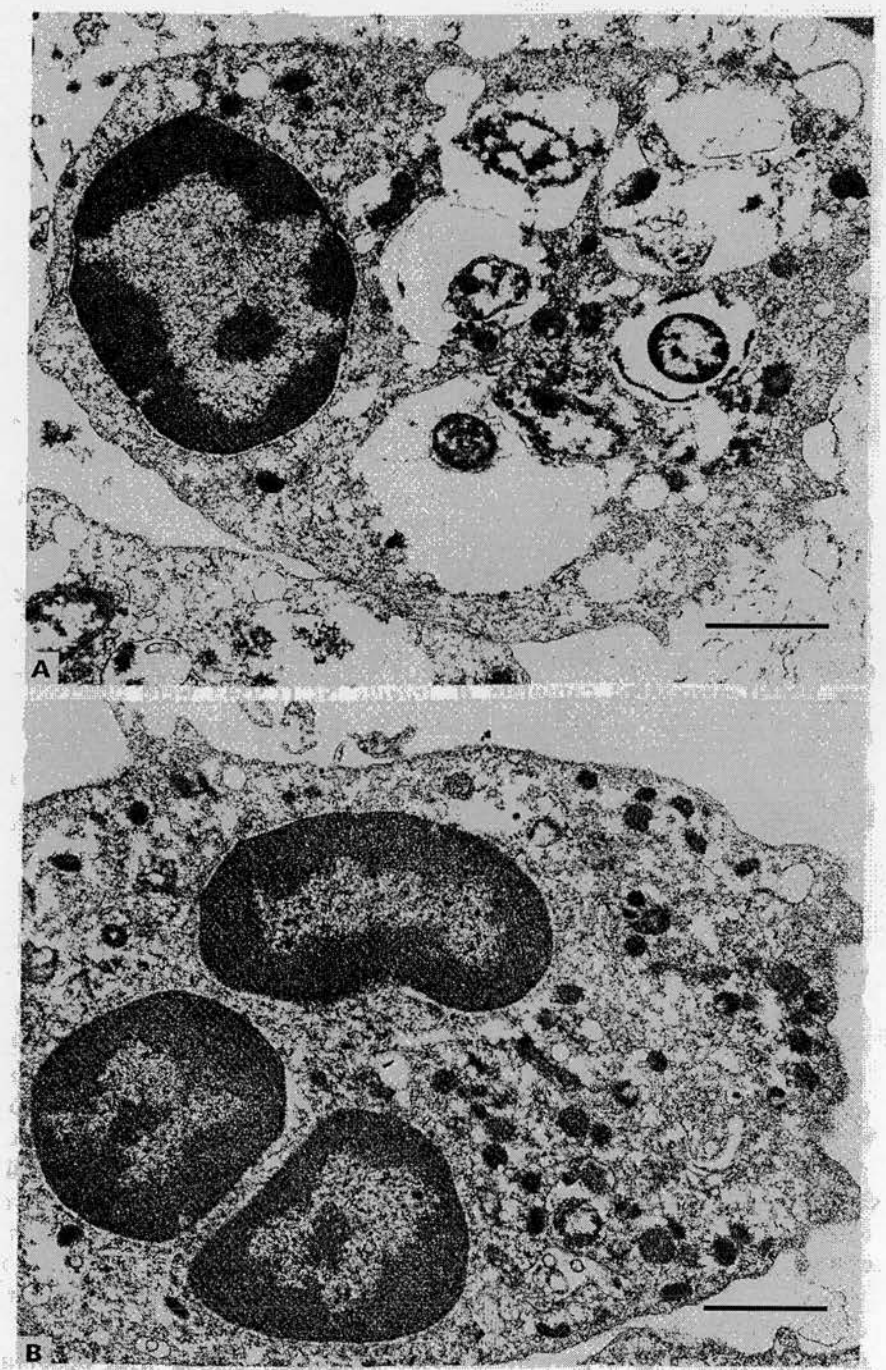

FIG. 5.—Electronmicrographs of PMNL after incubation for 60 min with (A) non-capsulate and (B) capsulate *B. fragilis* NCTC9343 in the presence of 10% serum. Scale bar = 1 μm.

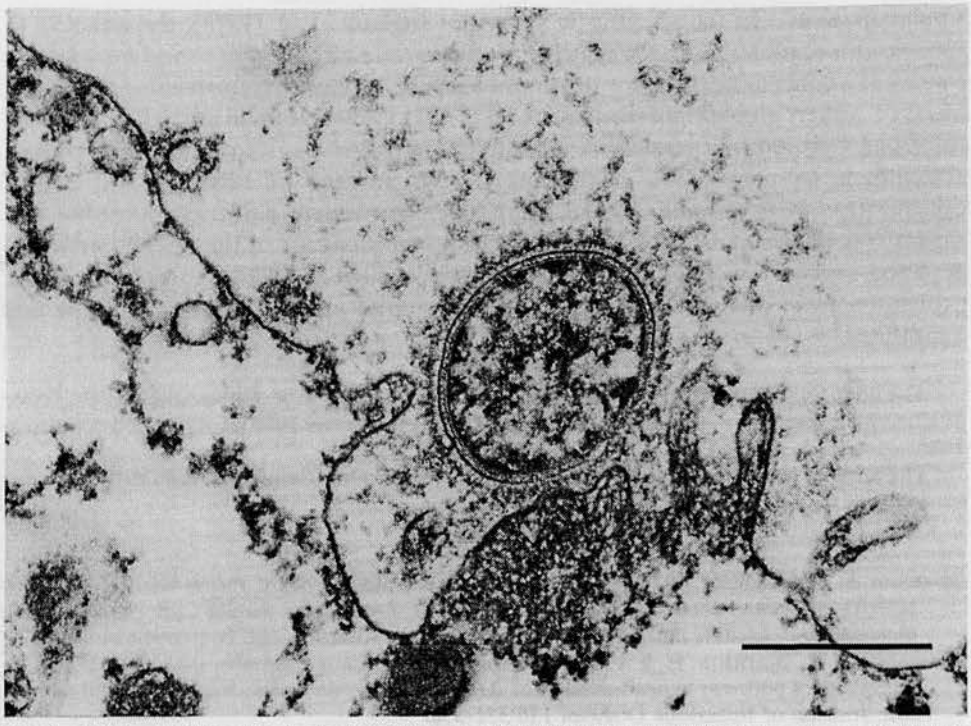

FIG. 6.—Electronmicrograph of capsulate *B. fragilis* NCTC9343 associated with the membrane of a PMNL after incubation for 60 min in the presence of 10% serum. Scale bar = 0·5 μm.

The capsulate variant of *B. fragilis* NCTC9343 grown in basal broth produced small capsules and was successfully phagocytosed at bacterial concentrations of 1×10^6 and 1×10^7 cfu/ml; this suggests that the quantity of capsular material is important in impairing phagocytosis. A reduction in the quantity of opsonin required to completely cover the surface of organisms grown in basal broth could account for this observation.

Our results suggest that capsulate and non-capsulate *B. fragilis* have different opsonic requirements; this might explain the difference between our results and those obtained by other workers. Bjornson and Bjornson (1978) and Bjornson, Bjornson and Kitko (1980) demonstrated that the alternative complement pathway and IgM were essential for phagocytosis; however, Tofte *et al.* (1980) detected opsonisation at a reduced rate in the absence of the classical complement pathway and antibody. The proportions of capsulate and non-capsulate bacteria used in their studies were not defined. The inconsistency in these results might be explained if phagocytosis experiments were repeated with homogeneous suspensions of capsulate and non-capsulate *B. fragilis*.

Ingham *et al.* (1977 and 1981) observed that phagocytic killing was impaired when *P. mirabilis* and high concentrations of *B. fragilis* (1×10^7 cfu/ml) were mixed with serum and PMNL *in vitro*. *B. fragilis* cells with large capsules might be responsible for the inhibitory effect. Horwitz and Silverstein (1980) reported that anti-capsular antibody and complement were essential for opsonisation and phagocytosis of capsulate *Escherichia coli*. Opsonisation of capsulate *B. fragilis* might proceed in a

similar manner. In the phagocytic system of Ingham *et al.* (1977), the quantity of specific anti-capsular antibody may be insufficient to completely opsonise capsulate *B. fragilis* at a concentration of 1×10^7 cfu/ml because normal human serum contains low levels of bacteroides-specific antibodies (Hofstad, 1979). If these partially opsonised capsulate cells induced premature degranulation of PMNL lysosomes as a result of attachment to the PMNL membrane in the absence of phagocytosis, and if opsonisation of *P. mirabilis* by the alternative complement pathway proceeded at a slower rate (Tofte *et al.*, 1980), the bactericidal mechanisms of the PMNL would be depleted before phagocytosis of *P. mirabilis* occurred. This could explain the protection from phagocytic killing. Further studies are needed to investigate this hypothesis.

The authors dedicate this paper to the memory of Professor R.R. Gillies who was Professor of Clinical Bacteriology at the Queen's University of Belfast from 1976 until his sudden death in 1983.

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Investigation of antigenic expression of *Bacteroides fragilis* by immunogold labelling and immunoblotting with a monoclonal antibody

(Non-homogeneous labelling; Western blot)

J.H. Reid, S. Patrick *, E. Dermott *, A. Trudgett * and S. Tabaqchali

Department of Medical Microbiology, St Bartholomew's Hospital Medical College, London, EC1A 7BE, and
** Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast, BT12 6BN, U.K.*

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1. SUMMARY

Electron microscopy and immunogold labelling with monoclonal antibody (McAb) Bfl identified an antigen expressed on some in vitro and in vivo grown *Bacteroides fragilis* NCTC9343 cells.

Immunoprecipitation with this McAb was used to enrich for *B. fragilis* NCTC9343 cells expressing the Bfl antigen. The McAb Bfl bound to an epitope close to the surface of the outer membrane, but the fibrous capsular network radiating from the bacterial surface was not labelled. Analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting identified 3 high-M_r bands which resisted heating and protease digestion but were partially sensitive to sodium periodate treatment.

2. INTRODUCTION

Surface antigens play an important role in bacterial virulence [1]. Since variation in antigenic expression may occur during in vitro subculture [2] it is essential to define the antigens expressed by bacteria before using them in pathogenicity studies. *B. fragilis* is the most common anaerobic organism isolated from clinical specimens [3]; however, little is known about the organisation of the surface antigens. A number of reports indicate that changes in bacteroides surface antigens occur during in vitro subculture. Variation in capsulation of *B. thetaiotaomicron* was reported by Burt et al. [4]. Kasper et al. [5] identified a surface polysaccharide which showed variation during in vitro and in vivo growth. Non-homogeneous expression of a heat-labile antigen by populations of the same *B. fragilis* strain was reported by Schwan et al. [6]. Patrick and Reid [7] observed that capsulate *B. fragilis* separated on a Percoll density gradient gradually reverted to the non-capsulate form during successive in vitro subcultures.

In this study antigenic expression during in vivo and in vitro growth of *B. fragilis* was investigated using a monoclonal antibody McAb Bfl. Light microscopy observations were made using immunofluorescence, and the antigen was located on the bacterial surface by electron microscopy and immunogold labelling. The nature of the antigen was investigated using SDS–PAGE and immunoblotting.

3. MATERIALS AND METHODS

3.1. Bacterial growth conditions

B. fragilis strains were grown to late exponential phase in defined broth [8] at 37°C in anaerobic jars using the procedure of Collee et al. [9], or in an anaerobic chamber (Forma Scientific) with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Identification was confirmed using the API 20A system.

3.2. Production of monoclonal antibodies

A BALB/c mouse was immunized with *B. fragilis* NCTC9343 whole cells. The mouse was inoculated intraperitoneally (i.p.) each week for 3 weeks with 0.2 ml of bacterial suspension, and an additional inoculation was given 4 days prior to the fusion. Spleen cells from the immunized mouse were fused with NSl-Ag4/1 myeloma cells using the polyethylene glycol fusion technique [10]. Hybrid cell lines were selected in RPMI1640 medium containing hypoxanthine–aminopterin–thymidine and 15% fetal calf serum (Gibco). Culture supernatant was screened for antibody to *B. fragilis* NCTC9343 by enzyme-linked immunosorbent assay (ELISA). Hybridoma supernatant was used in all experiments.

3.3. Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was used to screen for McAb producing hybridoma cell lines. Wells of PVC microtiter plates (Becton and Dickenson) were each coated with bacteria (1×10^6 cells $\cdot$ ml^{-1}) suspended in 50 mM carbonate buffer, pH 9.6. The plates were incubated at 37°C for 2 h and washed 3 times with phosphate-buffered saline, pH 7.4 (PBS) containing 0.05% Tween-20. Plates were stored at −20°C.

Hybridoma culture supernatant (100 μl) was added to each well, the plates were incubated at 37°C for 1 h and washed as before (dilutions if required were made in 1% BSA–PBS). Goat anti-mouse IgG-alkaline phosphatase conjugate (Tago) diluted 1 : 3000 in 1% BSA–PBS was added to each well (100 μl) and the plates were incubated at 37°C for 1 h. After washing, 100 μl *p*-nitrophenyl phosphate solution (1 mg $\cdot$ ml^{-1}) (Sigma) in 50 mM carbonate buffer, pH 9.8, containing 1 mM MgCl, was added to each well. The plate was incubated at 37°C for 1 h; the reaction was stopped with 100 μl 3 M NaOH and absorbance at 405 nm was read using a Dynatech automatic plate reader.

3.4. Enrichment for bacterial variants

Capsulate and non-capsulate *B. fragilis* (defined by light microscopy) were separated by Percoll density gradient centrifugation [7].

Bacteria expressing the surface antigen recognised by McAb Bfl were enriched using immunoprecipitation. Organisms harvested by centrifugation at 2500 × *g* for 15 min were washed once in Ringer solution (RS). Cells were resuspended in Bfl hybridoma culture supernatant and mixed for 60 min at room temperature. Bacteria were washed 3 times and resuspended in 1 ml RS. Sepharose beads coated with goat anti-mouse IgG (Sigma) were washed in sterile RS and added to the bacterial suspension. Beads were recovered by sedimentation at 1 × *g* for 10 min and gently washed 3 times in RS. The beads were resuspended in 20 ml of defined medium and incubated anaerobically at 37°C. Stock cultures were prepared by dispersing 1-ml aliquots and freezing at −70°C.

3.5. Preparation of antigen

Bacteria were extracted by the aqueous phenol method of Westphal and Luderitz [11]. The aqueous phase was centrifuged at 10 000 × *g* for 30 min at 4°C to remove insoluble material, and the supernatant was lyophilised and stored at −20°C. Bacteria were also extracted with a glycine buffer, pH 5.0, containing EDTA. Bacteria harvested from defined broth culture (500 ml) were resuspended in 10 ml of 3 mM glycine buffer pH 5.0 containing 10 mM EDTA and incubated at 37°C for 30 min. Whole cells were removed by centrifugation at 10 000 × *g* for 30 min at 4°C and the extract was stored at −20°C. Extracts were treated with protease or sodium periodate by the method of Cousland and Poxton [12].

3.6. Carbohydrate assay

The carbohydrate concentration was determined by the method of Morris [13] with D-glucose as a standard.

3.7. SDS–PAGE

This was performed on 10% vertical slab gels using the Laemmli buffer system [14] at a constant current of 40 mA per gel. A standard protein mixture (Sigma) was used, with an M_r range of 116 000–14 000.

3.8. Immunoblotting and enzyme immunoassay

The method of Towbin et al. was used, with the following modifications [15]. Material was transferred to nitrocellulose membrane (Transblot TM Transfer Medium, BioRad) in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing 20% methanol at 350 mA for 1.5 h. After washing for 10 min in Tris-buffered saline (20 mM Tris–HCl, 500 mM NaCl, pH 7.5; TBS) the membrane strip containing the protein M_r standards was fixed with 1% acetic acid and stained with amido black. The rest of the membrane was placed in 3% bovine serum albumin (BSA–TBS) for 16 h at 4°C. It was then transferred into hybridoma culture supernatant, or an appropriately diluted antiserum, and incubated for 2 h at room temperature. After 3 washes in 0.025% Tween-20/TBS, the membrane was placed in goat anti-mouse IgG-alkaline phosphatase conjugate (Tago) diluted 1 : 3000 in 1% BSA–TBS and incubated for 1 h at room temperature. The membrane was washed 3 times in Tween-20/TBS and placed in a solution of Fast Red (Sigma) containing naphthol-AS-MX-phosphate (Sigma). Colour development took place between 5 and 15 min, and the reaction was stopped by washing in distilled water. All the above steps were performed with gentle agitation throughout.

3.9. Immunogold labelling and electron microscopy

Bacteria were washed twice in 0.1 M sodium cacodylate/HCl buffer, pH 7.2 (CB) and fixed in CB containing 2% paraformaldehyde and 0.1% glutaraldehyde for 1 h at 4°C. The cells were again washed in CB, dehydrated in graded alcohols and embedded in LR White resin. For gold labelling, ultrathin sections on nickel grids were treated with McAb Bfl followed by goat anti-mouse IgG 20-nm gold conjugate (Janssen Pharmaceuticals). The grids were washed in 0.1% BSA–TBS, pH 8.2, and rinsed in distilled water. They were then stained with uranyl acetate and lead citrate and examined with a Philips 301 transmission electron microscope.

3.10. Immunofluorescence microscopy

Bacteria were suspended in PBS, fixed on multitest slides (Flow Laboratories) and treated with McAb Bfl followed by rabbit anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma). Slides were then washed in PBS and examined under a 100 × objective with a Zeiss fluorescence microscope.

3.11. In vivo passage

Chambers were constructed and implanted in

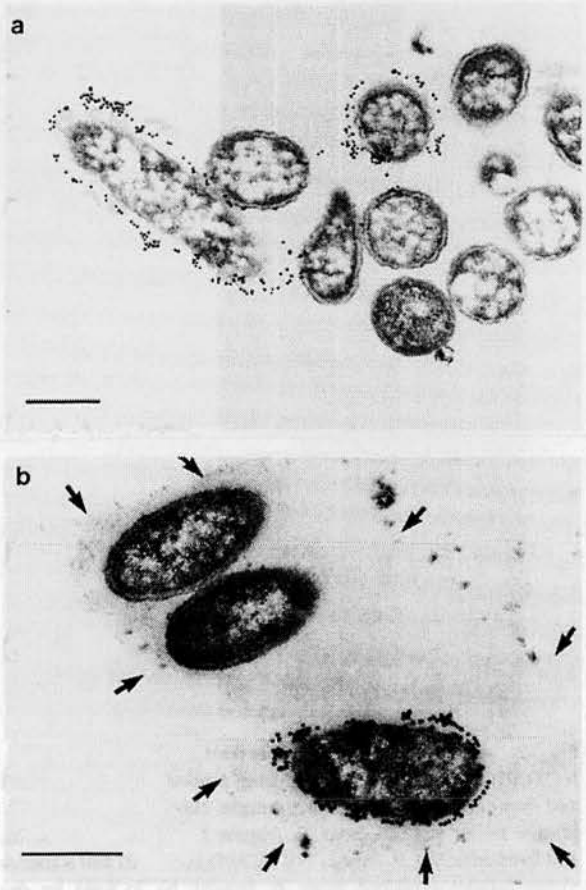

Fig. 1. Electron micrographs of in vitro-grown *B. fragilis* NCTC9343: (a) non-capsulate strain; (b) capsulate strain. Sections were treated with monoclonal antibody Bfl and labelled by the immunogold technique. Arrows indicate the capsule as a fibrous network. Scale bar = 0.5 μm.

the mouse peritoneal cavity as previously described [16].

4. RESULTS

Capsulate *B. fragilis* have an extracellular fibrous network which is absent on non-capsulate cells [17]. Electron microscopy and immunogold labelling with McAb Bfl identified an antigen close to the outer membrane on some cells of both populations, but the fibrous network was not labelled (Fig. 1). The proportion of labelled organism did not increase when bacteria grown in vivo were investigated (unpublished result).

Immunofluorescence was used to study reactivity with other *Bacteroides* strains. Of the 10 *B. fragilis* strains investigated, only one, a clinical isolate, had strong cross-reactivity.

Immunoprecipitation with McAb Bfl enriched populations for cells expressing the Bfl antigen. Normal and enriched populations tested in the API 20A system gave the same profiles. The Bfl antigen was extracted from cells using a glycine buffer pH 5.0 containing EDTA. Immunoblotting with McAb Bfl and mouse antiserum detected 3 diffusely stained high-M_r bands (Fig. 2, lanes a and c). Detection of antigen improved when extracts from enriched cultures were used (Fig. 2, lanes b and d). The antigen from enriched populations was resistant to digestion with protease; however, the intensity of the immunoblot was reduced when the antigen extract was treated with sodium periodate. The antigen resisted heating at 100°C for 3 min. No Bfl antigen was detected in the aqueous phase from a phenol–water extract by immunoblotting with either McAb Bfl (result not shown) or mouse antiserum (Fig. 2, lane e).

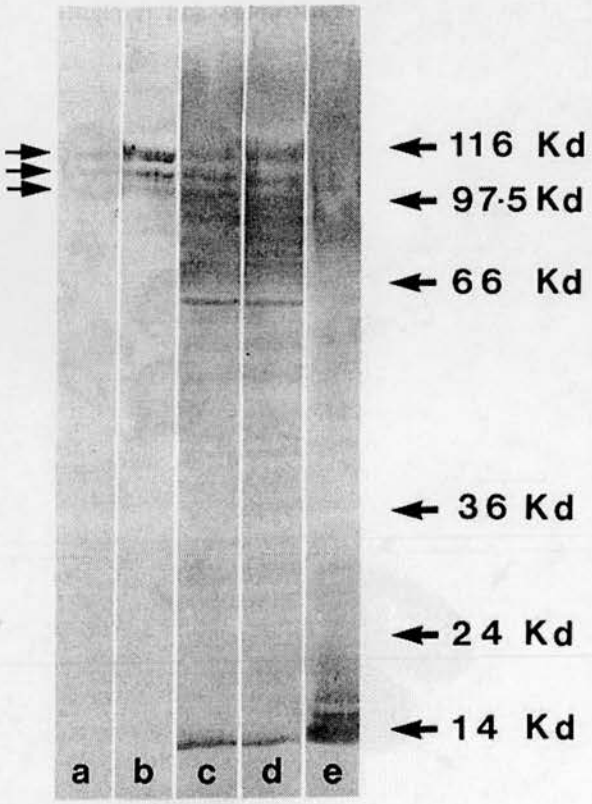

Fig. 2. Analysis of antigens extracted from *B. fragilis* NCTC9343 by immunoblotting using monoclonal antibody Bfl and immune mouse antiserum: antigen extracted with EDTA-glycine buffer pH 5.0 from *B. fragilis* NCTC9343 (a and c), and from enriched *B. fragilis* NCTC9343 (b and d) and aqueous phase antigen extracted from *B. fragilis* NCTC9343 by the phenol-water method (e). The concentrations of carbohydrate loaded were: 100 μg in lanes (a) and (b), 50 μg in lanes (c) and (d) and 15 μg in lane (e). Strips (a) and (b) were blotted with McAb Bfl and strips (c), (d) and (e) were blotted with mouse antiserum.

5. DISCUSSION

Immunogold labelling demonstrated that the the Bfl epitope was not expressed by all *B. fragilis* NCTC9343 cells, although no structure was observed by electron microscopy which could be associated with the labelling pattern. With the fixation and staining methods used in this study, capsulate cells had an extracellular fibrous network and non-capsulate cells no outer layers. A narrow electron-dense layer, adjacent to the outer membrane, has been observed on *B. fragilis* NCTC9343 by electron microscopy after fixation in osmium tetroxide and glutaraldehyde (unpublished result). The relationship between this layer and the Bfl epitope remains to be determined.

Schwan et al. [6] also observed a similar non-homogeneous staining pattern with 9 different strains of *B. fragilis* using immunofluorescence

and heterologous rabbit antiserum. This was related to the expression of an antigen susceptible to heating at 100°C for 30 min. The high M_r structure containing the Bfl epitope could be a polysaccharide or a lipopolysaccharide since it is resistant to protease digestion, boiling at 100°C for 3 min and partially sensitive to sodium periodate. The antigen was not detected in the aqueous phase of a phenol–water extract; however, the insoluble pellet removed from the aqueous phase by centrifugation at $10\,000 \times g$ could contain the Bfl epitope. Alternatively, the antigen could be sensitive to phenol treatment or soluble in the phenol phase. Some types of lipopolysaccharide extracted from *Citrobacter* strains were phenol-soluble [18]. Other techniques for extracting lipopolysaccharide are being investigated.

Immunoprecipitation with McAb Bfl is a useful method of enrichment for *B. fragilis* cells expressing the Bfl antigen. Repeated immunoprecipitation with McAb should produce homogeneous populations of bacteria expressing the antigen of interest. Bacteria not expressing the antigen remain in suspension and can also be recovered. Such homogeneous populations of bacteria could be used to determine the factors controlling the expression of surface antigens and to determine which antigens are involved in the virulence of *B. fragilis*.

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Capsulation of *in vitro* and *in vivo* Grown *Bacteroides* Species

By SHEILA PATRICK,* JOHN H. REID AND ALAN COFFEY

Department of Microbiology and Immunobiology, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK

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By centrifugation on a four step Percoll density gradient cells of *Bacteroides* species could be separated according to the size of extracellular structure. The difference in size was visible by both light and electron microscopy. Two structures were observed on *Bacteroides fragilis* by electron microscopy, namely a fibrous network and an electron dense layer. An electron dense layer was visible on *Bacteroides ovatus* only when stained with ruthenium red. *B. fragilis* cells grown in the mouse peritoneal cavity did not produce a large fibrous network. An electron dense layer was observed on some cells in the presence of ruthenium red stain and cells possessing this layer were phagocytosed *in vivo*.

INTRODUCTION

The obligate anaerobe *Bacteroides fragilis* is the Gram-negative anaerobic organism most commonly isolated from clinical infections, e.g. bacteraemia, wound, intra-abdominal and urogenital infections (Lindberg *et al.*, 1979). Capsules have been associated with the virulence of a number of bacteria (Robbins *et al.*, 1980) and the extracellular layers observed on *B. fragilis* may fulfil a similar function (Hofstad, 1984). Capsulation of *B. fragilis* and other *Bacteroides* species is subject to variation both in capsule size and in the number of cells producing capsules within a population (Babb & Cummins, 1978; Burt *et al.*, 1978; Lindberg *et al.*, 1979; Brook *et al.*, 1984). The factors influencing these variations have yet to be determined, although capsule size can vary with the growth medium (Patrick & Reid, 1983). Two extracellular structures have been observed by electron microscopy, a narrow electron dense layer close to the outer membrane (Kasper *et al.*, 1977; Lindberg *et al.*, 1979; Reid, 1983; Brook *et al.*, 1984) and a more extensive fibrous network (Patrick & Reid, 1983; Reid, 1983; Lambe *et al.*, 1984). It has been suggested that the difference in the appearance of these structures is related to the condensation of hydrated extracellular material which occurs during preparation for the electron microscope (Lambe *et al.*, 1984). However, these authors did not define the populations examined with respect to either the proportion of cells with extracellular structures or variation in the size of extracellular structure examined by wet India ink stain and light microscopy.

We have previously reported the separation of *B. fragilis* cells with different sizes of extracellular structures (or capsules) as defined by light microscopy and wet India ink negative stain (Patrick & Reid, 1983). We now report on the ultrastructure of these separated populations after electron microscopy and examine cells with initially large and small extracellular structures after 24 h growth in the mouse peritoneal cavity.

METHODS

Bacterial culture. B. fragilis (NCTC 9343) was supplied by the Department of Bacteriology, University of Edinburgh Medical School, *B. fragilis* (ATCC 23745) by the American Type Culture Collection, Rockville, Md, USA, and *B. ovatus* (ATCC 8483) and *B. fragilis* (NCTC 10584) were departmental stock cultures. Bacteria were

Abbreviations: EDL, electron dense layer; EM, electron microscopy; LM, light microscopy; OM, outer membrane; RR, ruthenium red.

grown in defined broth (van Tassell & Wilkins, 1978) and incubated at 37 °C in an atmosphere of H_2 (90%) and CO_2 (10%) in anaerobic jars. The standard anaerobic procedures of Collee *et al.* (1972) were used.

Separation of bacteria. Bacteria were grown to late exponential phase in defined broth and layered onto a Percoll (Pharmacia) discontinuous density gradient, centrifuged at 2600 *g* for 20 min in a bench centrifuge and the cells from each interface of the gradient were removed as previously described (Patrick & Reid, 1983).

Microscopy. Capsulation was determined by light microscopy with eosin–carbol fuchsin negative staining (Cruickshank, 1965). Bacteria were fixed for electron microscopy by washing and resuspending in 0·1 M-cacodylate buffer (pH 6·8) containing 2·5% (v/v) glutaraldehyde and incubated for 1 h at 4 °C in the dark. The bacteria were then washed in 0·1 M-cacodylate buffer containing 1% (w/v) osmium tetroxide and incubated at room temperature for 3 h in the dark. Where cultures were stained with ruthenium red (Springer & Roth, 1973) this was included at both fixation stages at a concentration of 1 mg ml^{-1}. Bacteria were then washed before dehydration in a graded series of alcohols as follows: 50%, 75%, 95% ethanol, absolute ethanol, water free absolute ethanol and finally two washings in propylene oxide. Each step lasted 10–15 min. Preparations were then embedded in Spurr resin and ultrathin sections were examined with a Philips 301 transmission electron microscope.

In vivo culture of bacteria. Chambers were constructed and implanted in the mouse peritoneal cavity as previously described (Patrick *et al.*, 1984). The chambers were constructed with either 0·45 μm pore membrane filters (Millipore), which excluded phagocytes, or 3 μm pore filters, which allowed their entry.

RESULTS

Light microscopy

Light microscopy (LM) of *Bacteroides* species stained negatively with wet India ink or eosin–carbol fuchsin showed a range of capsule size, the largest being equivalent to the diameter of the cell (Patrick & Reid, 1983). Cells with the largest size of capsule were easily distinguished by LM and the proportion of cells with this structure apparently varied with the species and strain.

We separated cells with different capsule sizes on a four step Percoll gradient. Cells with large capsules were found at the 0–20% (top) interface, cells with intermediate sizes of capsules at the 20–40% and 40–60% interfaces and cells with no capsules by LM at the 60–80% (bottom) interface.

The pattern of bands observed with populations grown from laboratory stock cultures (normal populations) showed that *B. fragilis* NCTC 9343 and NCTC 10584 each had less than 1% of the bacterial population with large capsules. *B. fragilis* ATCC 23745 had 17% of cells with large capsules, whereas *B. ovatus* ATCC 8483 had 80% of cells with large capsules. Cells with

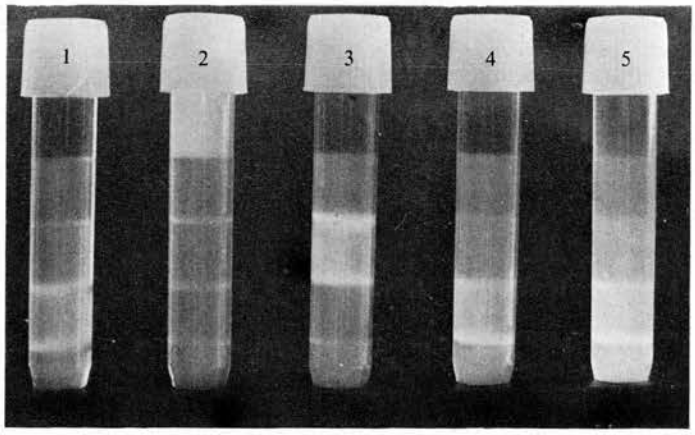

Fig. 1. Four step (20, 40, 60 and 80%) Percoll density gradients after centrifugation with *B. fragilis* NCTC 9343. Tube 1, pattern of bands when laboratory stock culture was centrifuged. Tubes 2–5, separation, on the same gradients, of cells from first subculture from each interface: 2, 0–20% (top) interface; 3, 20–40% interface; 4, 40–60% interface; 5, 60–80% (bottom) interface.

Table 1. *Incidence of fibrous network and electron dense layer in B. fragilis (NCTC 9343) and B. ovatus (ATCC 8483) after density gradient separation*

Results were obtained by electron microscopy after separation on a discontinuous Percoll gradient (see Methods): ++, extensive fibrous network; +, presence of structure; −, absence of structure.

	Gradient position						
	0–20% Interface		20–40% Interface		40–60% Interface*	60% Layer*	60–80% Interface*
Structure	*B. fragilis*	*B. ovatus*	*B. fragilis*	*B. ovatus*	*B. fragilis*	*B. ovatus*	*B. fragilis*
Fibrous network	++	++	+	+	+	−	−
Electron dense layer	+	−	+	+/−	+	−	+
Fig.	2(*b*, *d*)	2(*a*, *c*)	4(*a*)	3(*a*, *b*)	4(*b*)	5	6

* *B. ovatus* cells remained in the 60% Percoll layer and did not accumulate at either the 40–60% or the 60–80% interface.

intermediate capsules were present in all cultures except *B. fragilis* ATCC 23745. If cells from the different interfaces were each subcultured in defined medium, the populations could be enriched to some extent for the cells that predominated at that interface. The results for *B. fragilis* NCTC 9343 are illustrated in Fig. 1. The identity of bacterial suspensions taken from the Percoll interfaces of the enriched cultures was confirmed with the API 20A identification system and suspensions were streaked out for single colonies to confirm the purity of the cultures. Therefore the distribution of bacterial cells on the gradients could alter depending on the culture inoculum and there was a degree of stability in capsule size during the first subculture.

Electron microscopy of cells grown in vitro

The results are summarized in Table 1.

Cells of *B. fragilis* and *B. ovatus* with large capsules, as defined by LM (0–20% Percoll interface), showed an extensive fibrous network which had a coarser appearance when stained with ruthenium red (RR) (Fig. 2). *B. fragilis* cells also had an electron dense layer (EDL) adjacent to the outer membrane (OM) (Fig. 2*b*, *d*).

Cells with less capsular material by LM (20–40 and 40–60% Percoll interfaces) had either a marginal fibrous network or an EDL (Figs 3 and 4). In the case of *B. ovatus* an EDL was observed only when stained with RR (Fig. 3).

Cells which lacked capsular material by LM (60–80% Percoll interface) had no extracellular structures in the case of *B. ovatus*; however, an EDL was present in *B. fragilis* (Figs 5 and 6).

Therefore the range of capsule sizes observed by LM was also observed by EM and *B. fragilis*, defined as noncapsulate by LM, had an extracellular EDL.

EM of B. fragilis grown in vivo

B. fragilis taken from the 0–20% (Fig. 2) and 60–80% (Fig. 6) Percoll interfaces was grown for 24 h in chambers implanted in the mouse peritoneal cavity (Patrick *et al.*, 1984). The chambers were constructed either to exclude or to allow the entry of phagocytic cells. EM indicated that neither bacterial population produced large amounts of the fibrous network *in vivo*. Cells had either small amounts of fibrous network or a narrow EDL or no outer layer, both in the presence (Fig. 7*a*, *b*) and absence (Fig. 8*a*, *b*) of phagocytes. Where phagocytes entered the chamber, it was apparent that cells with and without the EDL were phagocytosed (Fig. 7*a*, *b*).

All the results shown are representative of at least three replicate experiments.

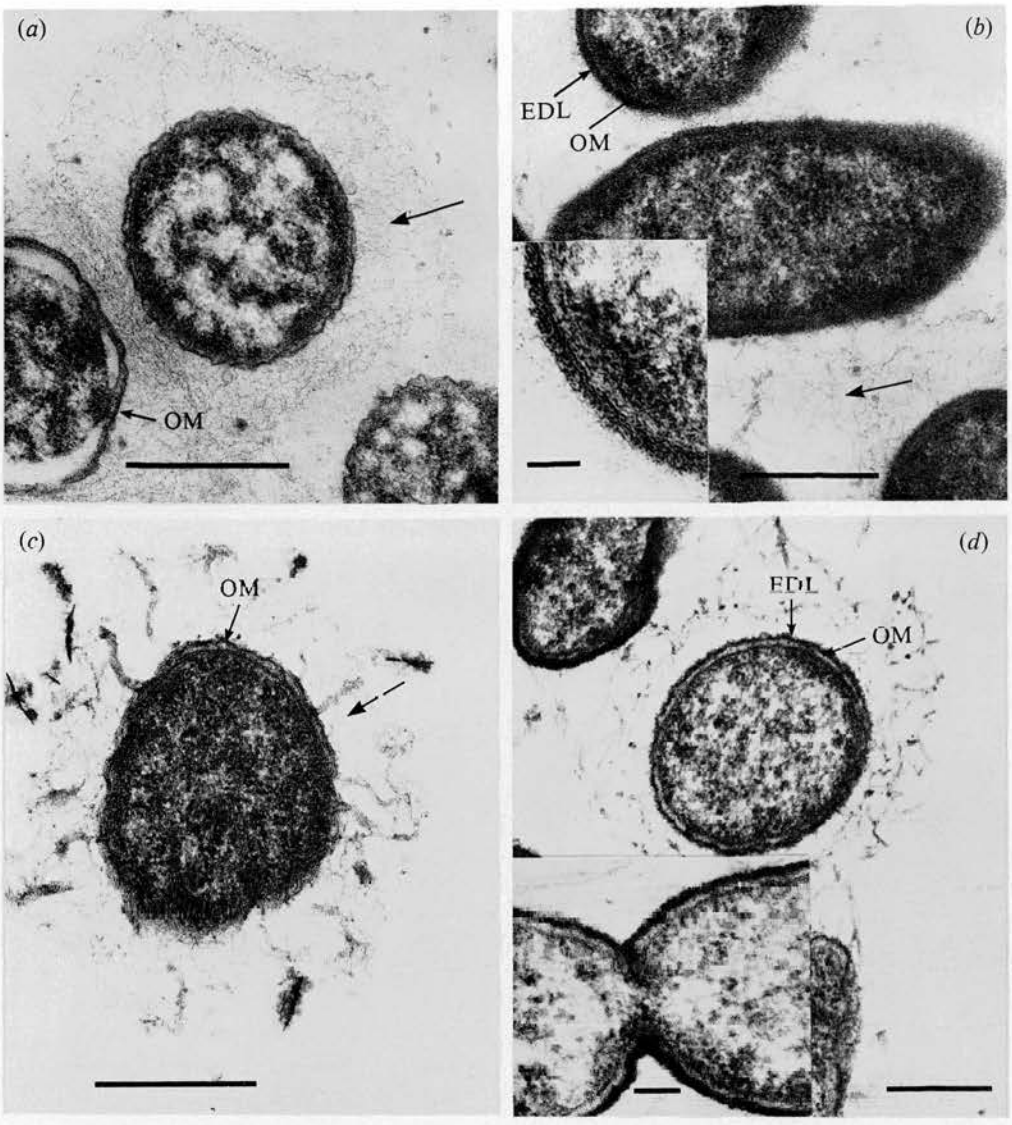

Fig. 2. Electron micrographs of cells from the 0–20% Percoll interface to illustrate the fibrous network (arrowed) on *B. ovatus* ATCC 8483 (*a*) and *B. fragilis* ATCC 23745 (*b*), and the electron dense layer (EDL) adjacent to the outer membrane (OM) of *B. fragilis* ATCC 23745, visible without ruthenium red (RR) stain. Note the coarser appearance of the fibrous network after RR staining on *B. ovatus* ATCC 8483 (*c*) and *B. fragilis* ATCC 23745 (*d*). Bar markers, 0·5 μm in micrographs and 100 nm in insets showing detail of the outer layers. Abbreviations are the same for all figures.

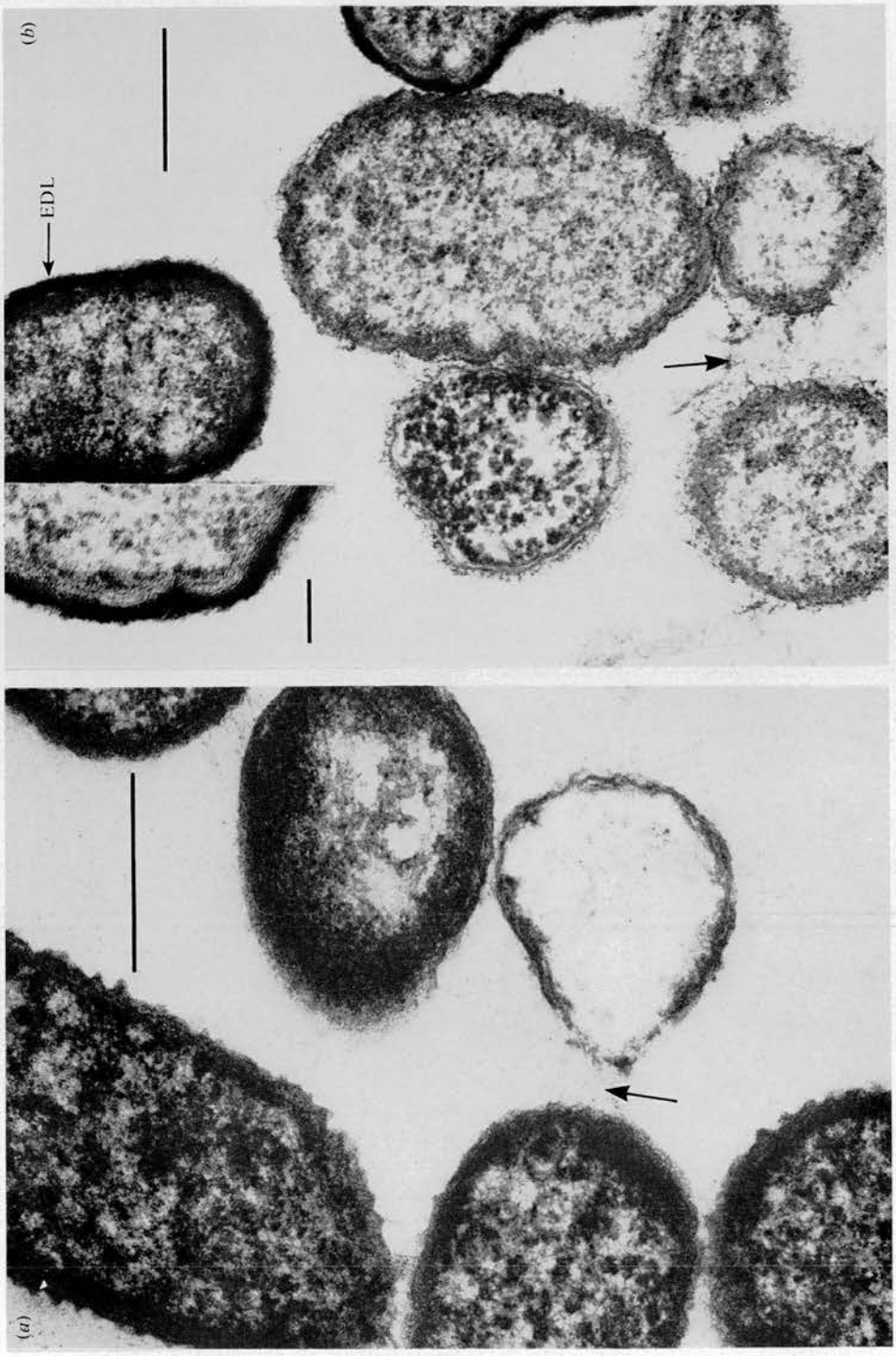

Fig. 3. Electron micrographs of *B. ovatus* ATCC 8483 from the 20–40% Percoll interface to illustrate the small amounts of fibrous network (arrowed) visible in the absence of RR stain (*a*) and the EDL and fibrous network observed after RR staining (*b*). Bar markers, see Fig. 2.

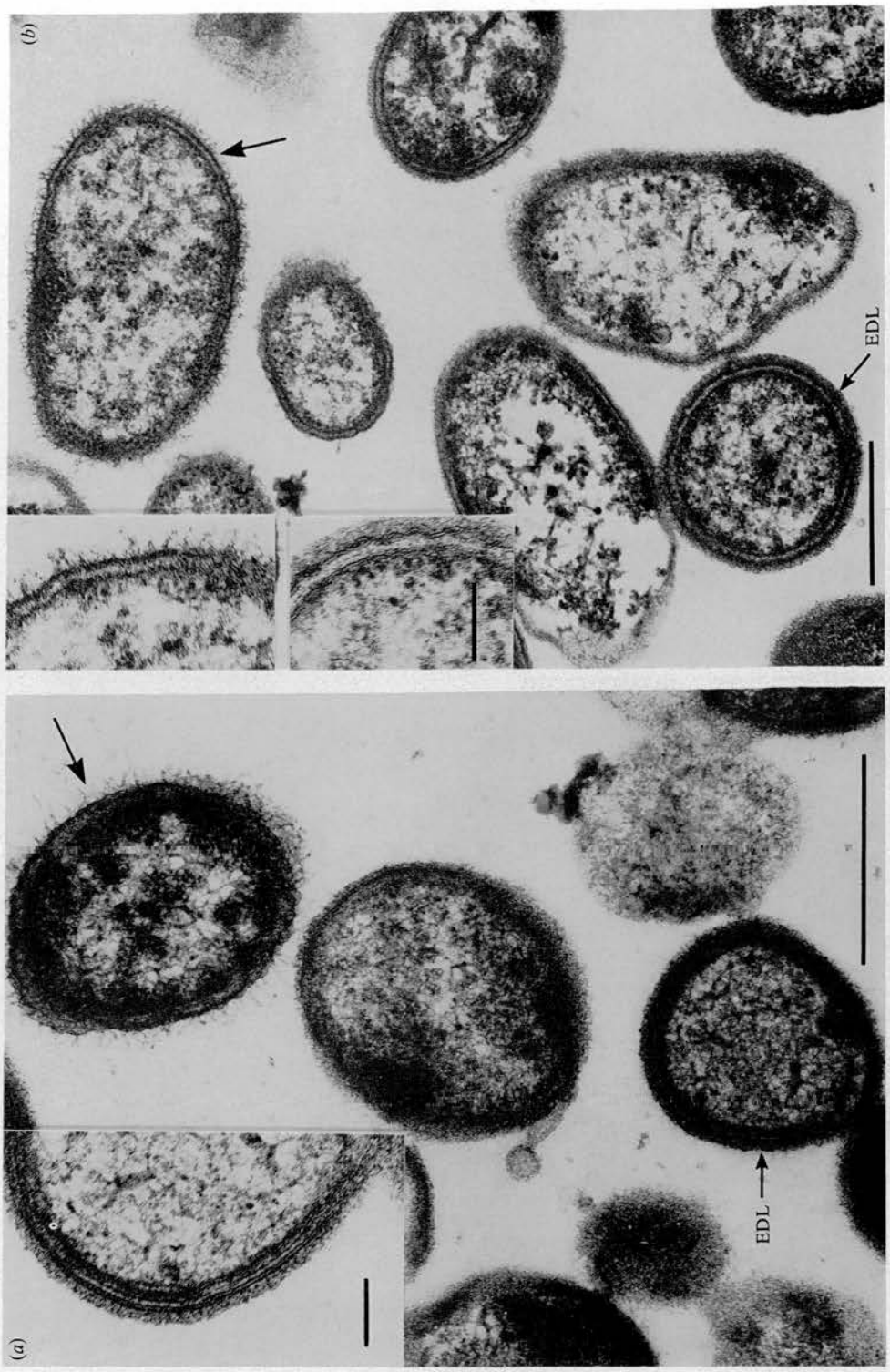

Fig. 4. Electron micrographs of *B. fragilis* NCTC 9343 from the 20–40% Percoll interface, not stained with RR (*a*) and from the 40–60% Percoll interface, stained with RR (*b*). Note that cells have either an EDL or a small fibrous network and that these are both visible in the absence of RR stain. Bar markers, see Fig. 2.

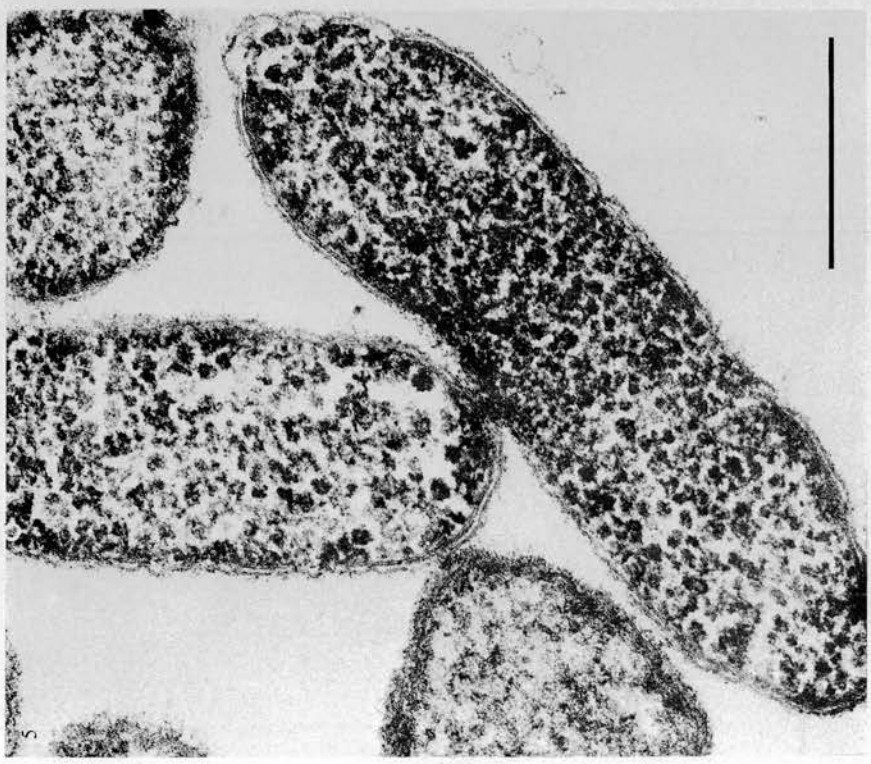

Fig. 5. Electron micrograph of *B. ovatus* ATCC 8483 from the 60% Percoll layer stained with RR. Note the absence of an EDL. Bar markers, see Fig. 2.

Fig. 6. Electron micrograph of *B. fragilis* NCTC 9343 from the 60–80% interface without RR stain. Note than an EDL is present on some cells. Bar markers, see Fig. 2.

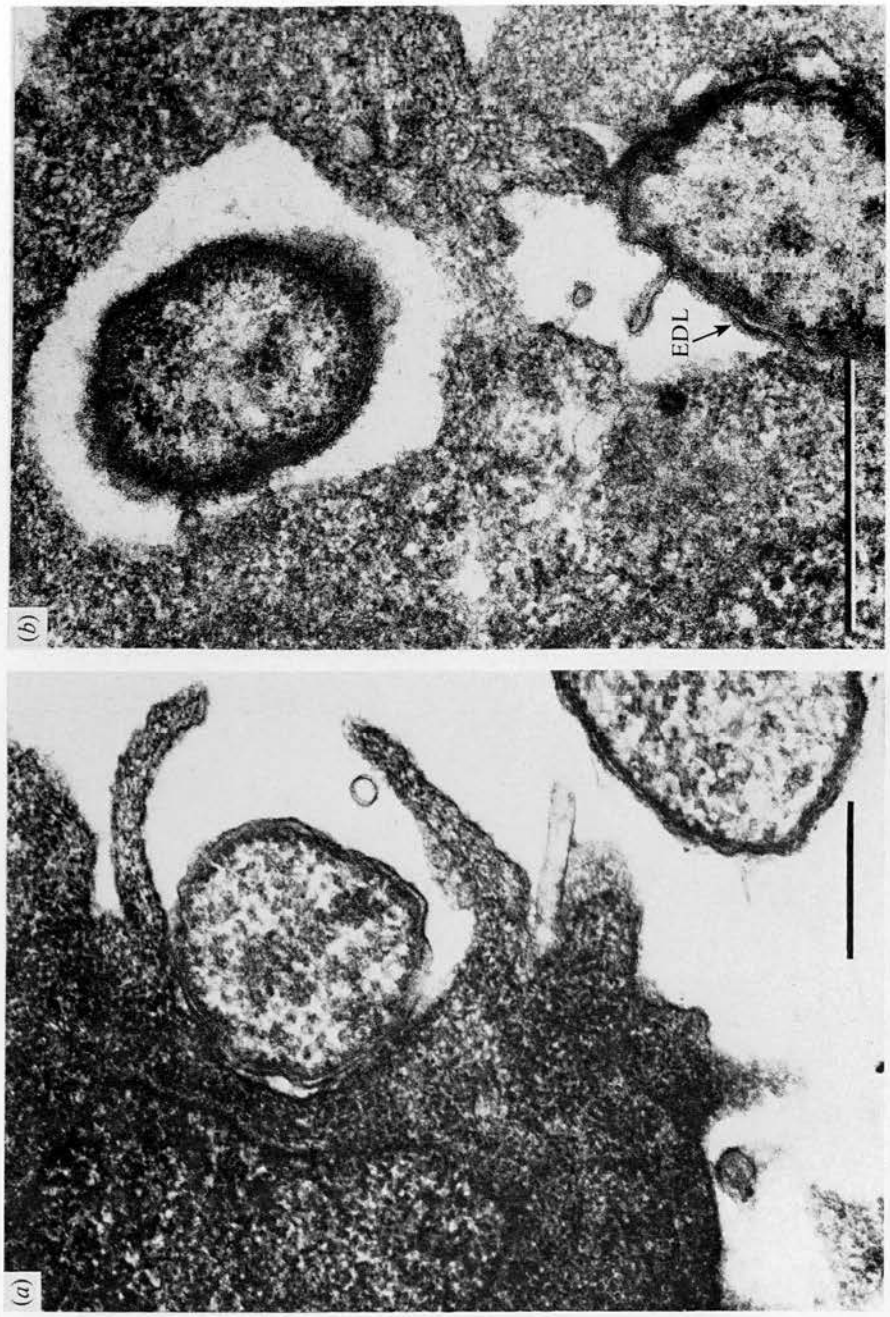

Fig. 7. Electron micrographs of *B. fragilis* NCTC 10584 after incubation for 24 h in the mouse peritoneal cavity in the presence of phagocytes. Inoculum taken from the 0–20% Percoll interface. Note the absence of any extensive fibrous network and the presence of an EDL on a cell apparently inside a phagocytic vacuole (*b*). Bar markers, see Fig. 2.

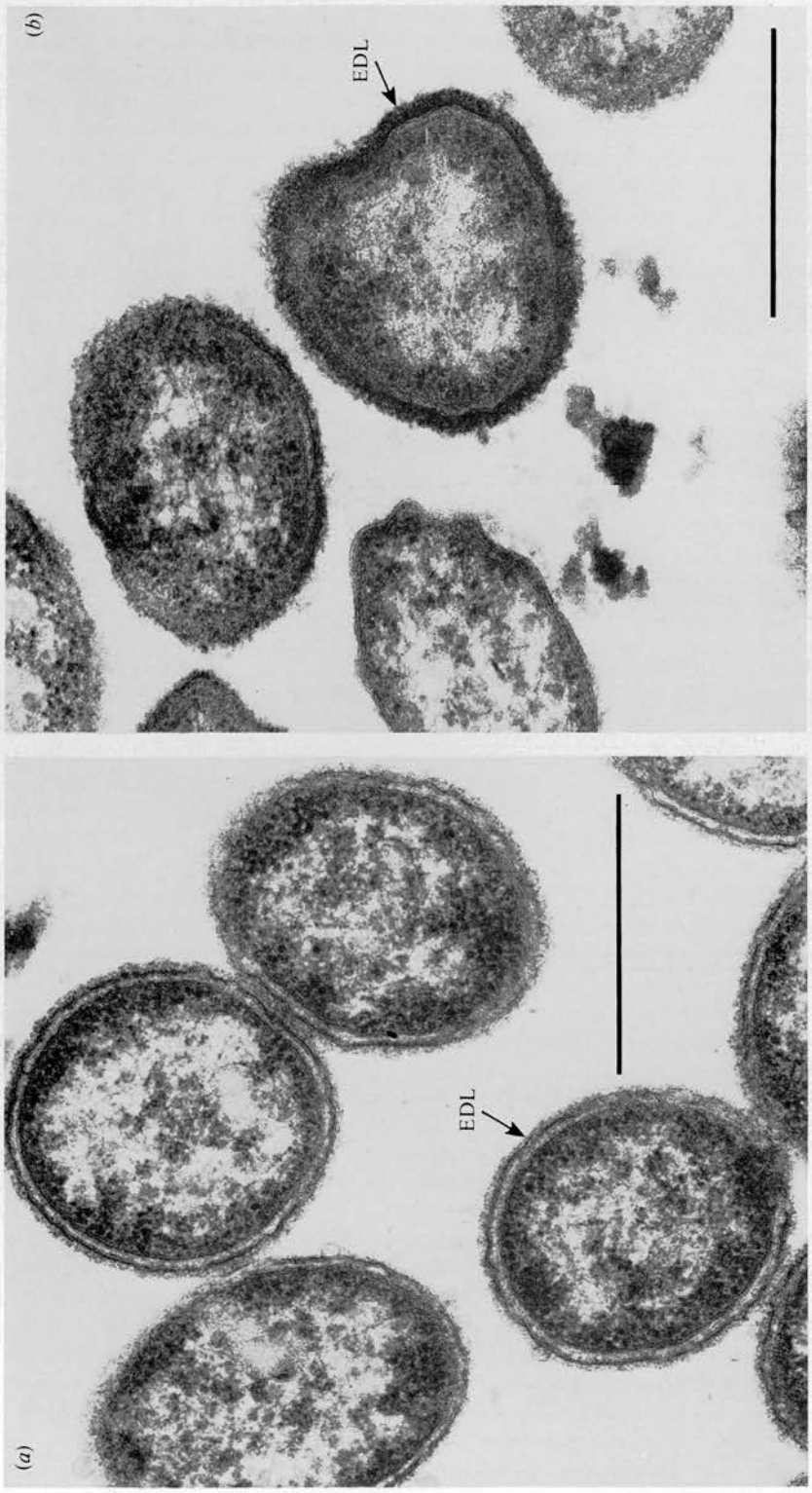

Fig. 8. Electron micrographs of RR stained *B. fragilis* NCTC 9343 after incubation for 24 h in the mouse peritoneal cavity in the absence of phagocytes. Inoculum taken from (*a*) the 0–20% Percoll interface and (*b*) the 60–80% Percoll interface. Note the absence of any extensive fibrous network. Bar markers, see Fig. 2.

DISCUSSION

Some of the structural differences in capsular material observed in the present study reflect different degrees of condensation with RR stain (Fig. 2); in the case of *B. ovatus* (Fig. 3) electron dense material was observed only with RR stain. Lambe *et al.* (1984) suggested that the fibrous network or glycocalyx condensed to form an EDL where it was not connected to more than one cell. However, an EDL and a marginal fibrous network were observed on adjacent cells of *B. fragilis* (Fig. 4), which by LM had similar sizes of capsules. Also, *B. fragilis* from the 60–80% Percoll interface lacked any capsule by LM, yet possessed an EDL by EM. Therefore it is possible that in some cases the EDL and fibrous network are different in nature. The ability of the cells to produce these different sized capsules is a relatively stable trait because subculture from the Percoll interfaces enriched populations for a particular size of capsule (Fig. 1).

An EDL of approximately 35 nm has been observed outside the OM in clinical isolates of *B. fragilis* and some strains of *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* by EM after RR staining (Kasper *et al.*, 1977, 1979). Similar structures have been reported by Lindberg *et al.* (1979) and Brook *et al.* (1984), but none of these authors reported the presence of fibrous material. Babb & Cummins (1977) observed capsules one to four times the diameter of the cell by negative stain and LM. These authors considered it likely that this structure differed from the RR stained layer described by Kasper *et al.* (1977). These anomalies could be due to differences in the composition of the culture media (Patrick & Reid, 1983). However, some of the pili-like structures observed by Brook *et al.* (1984) in thin sections could be coarse fibrous network similar to that observed after RR staining of cells from the 0–20% interface (Fig. 2*c*, *d*).

B. fragilis ATCC 23745, which possessed the RR staining layer, was reported to induce abscesses in a rat model of intra-abdominal infection, whereas *Bacteroides* species lacking this layer (e.g. *B. vulgatus*) did not. A crude preparation of polysaccharide material from strain ATCC 23745, which was thought to correspond to the RR staining layer, produced abscesses in the rat model in the absence of live bacteria (Onderdonk *et al.*, 1977). Subsequent investigations, however, indicated that this crude extract contained both capsular material and lipopolysaccharide (Kasper *et al.*, 1983). It was therefore unclear which of the outer layers of *B. fragilis* were the important virulence determinants. In the present study, an EDL was observed on some of the organisms grown *in vivo*. Large amounts of fibrous material were not produced in the mouse model of infection, although some of the cells retained the ability to do so. We have previously shown that after subculture in defined medium the fibrous network reappeared on up to 40% of the 0–20% interface cells grown *in vivo* but on less than 1% of the 60–80% interface cells (Patrick *et al.*, 1984). The lack of fibrous network *in vivo* may relate to the nutrients available in the intraperitoneally implanted chambers. It remains to be seen, however, why a population with the ability to produce a fibrous network is not selected, as studies *in vitro* indicate that the fibrous network confers resistance to phagocytosis and this resistance is related to the total amount of fibrous network present; preparations from the 60–80% interface were phagocytosed *in vitro* (Reid & Patrick, 1984). It is interesting that populations of *B. fragilis* NCTC 9343 from the 0–20% and 60–80% interfaces were resistant to killing by 20% normal human serum whereas *B. ovatus* ATCC 8483, which lacked an EDL in the absence of RR stain, was susceptible (Reid, 1983). Cells possessing an EDL were phagocytosed *in vivo*, but these layers (Figs 7 and 8) cannot be related to those observed on cells grown *in vitro* (e.g. Fig. 5) as material from the mouse may adhere to the bacterial surface.

Clearly it is important to determine the role of the 'classical' capsule of *Bacteroides* species (defined by wet India ink negative staining) in the pathogenicity of these organisms and also its relationship to the EDL observed by EM. Immunoelectron and immunofluorescence microscopy with monoclonal antibodies indicate that *B. fragilis* populations are antigenically heterogeneous (Reid *et al.*, 1985) and a monoclonal antibody with an epitope which is enriched in cells from the 20–40% interface has been characterized (unpublished result). It therefore seems that structural differences observed by EM are reflected by differences in antigenicity.

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Screening for the presence of the insertion sequence IS1 in the genus *Bacteroides*

(*Bacteroides* sp.; insertion sequence IS1)

M.J. Larkin, G.W. Blakely, D.R. Williams and S. Patrick *

*Sub-Department of Microbiology, The Queen's University of Belfast, David Keir Building, Belfast BT7, 1NN, and * Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast, BT12 6BN, U.K.*

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1. SUMMARY

A specific DNA probe, containing a conserved region of the insertion sequence IS1, was hybridised to dot blots of total genomic DNA from 2 oral and 5 intestinal *Bacteroides* spp. Using *Escherichia coli* K12 as a positive control and *Pseudomonas aeruginosa* as a negative control, DNA homologous to the probe could not be detected in *Bacteroides corporis, Bacteroides intermedius, Bacteroides ovatus, Bacteroides vulgatus, Bacteroides thetaiotaomicron* or 2 strains of *Bacteroides fragilis.* The total DNA included plasmid DNA of 30.2, 42.7 and 42.7 MDa from *B. fragilis, B. intermedius* and *B. corporis,* respectively.

IS1 is commonly found in members of the Enterobacteriaceae, and it was concluded that the 2 groups of bacteria are not closely related.

2. INTRODUCTION

Insertion sequences are a class of short transposable elements which only encode determinants associated with their own transposition [1]. They promote a number of genetic changes which include insertions, deletions, inversions, duplications and replicon fusions. They are considered to be normal constituents of bacterial genomes and responsible for many spontaneous mutations. They also provide portable regions of homology for homology-dependent recombination mechanisms and may be the foci for major gene rearrangements during evolution [2]. The insertion sequence IS1 was originally discovered as the cause of strong polar mutations in the galactose operon of *E. coli* K12 [3]. Determination of its base sequence of 768 bp has shown it to be the shortest functional insertion sequence [4], and 2 open reading frames within the element are conserved and required for transposition [5,6].

A number of studies have shown that IS1 is commonly present in the genomes of Enterobacteriaceae. For example, there are 6–10 copies of IS1 in the genome of *E. coli* K12 [7,8], 4–19 in *E. coli* B [9], 3 in *E. coli* C [8], more than 40 in *Shigella dysenteriae* and *Shigella flexneri,* 2 in *Shigella boydii,* 1 in *Klebsiella aerogenes* [8], and 1 or 2 in *Serratia marcescens* [8,10]. Other related species such as *E. coli* W, *Salmonella typhimurium* and *Proteus mirabilis* have either none or only one copy [8,11], and Ohtsubo et al. [12] concluded that the evolution of IS1 did not take place at the same

time as the divergence of most of the bacterial DNA. More distantly related species, such as *P. aeruginosa* and *Bacillus subtilis* have no copies of IS1 [10]. *Bacteroides* spp. have not been screened for the presence of IS1 before, although they are closely associated with members of the Enterobacteriaceae in the mammalian intestinal tract.

In this study, 2 oral and 5 intestinal *Bacteroides* spp. were screened for the presence of plasmid DNA and DNA homologous to IS1.

3. MATERIALS AND METHODS

3.1. Organisms

The oral *Bacteroides* spp used were; *B. intermedius* NCTC9336 and *B. corporis* ATCC33545. The intestinal *Bacteroides* spp were; *B. ovatus* ATCC8463, *B. vulgatus* NCTC10583, *B. thetaiotaomicron* NCTC10582, *B. fragilis* NCTC9343 and *B. fragilis* JC19 (a 1984 clinical isolate from the Royal Victoria Hospital, Belfast). *E. coli* K12 NCIB10083 and *P. aeruginosa* 8602 [13] were used as positive and negative controls, respectively. All bacteria were maintained at −70°C. Identification of the *Bacteroides* spp. was confirmed using the API 20A system.

3.2. Media

The *Bacteroides* spp. were initially grown on 5% human lysed-blood agar and then on basal broth medium [14] in anaerobic jars at 37°C as detailed by Patrick and Reid [15]. Cultures were incubated aerobically to check for aerobic contaminants. The other bacteria were grown aerobically at 37°C on nutrient agar and nutrient broth (Oxoid).

3.3. Extraction of DNA and dot blotting

Cells from 500 ml of broth culture were harvested by centrifugation (10 000 × *g* for 15 min), resuspended in 10 ml 0.1 M Tris-HCl buffer, pH 8.0, lysed with lysozyme for 90 min at 37°C as described by Maniatis et al. [16], and 1.2 ml 20% (w/v) SDS was added and the mixture was incubated for a further 20 min. Protein was removed by repeated extraction with equal volumes of chloroform until the aqueous phase was almost clear. An equal volume of cold absolute ethanol was added, the DNA spooled out, dissolved in 10 ml of SSC buffer (0.87 $g \cdot l^{-1}$ sodium chloride and 0.44 $g \cdot l^{-1}$ sodium citrate, pH 7.0) and treated with RNase (Sigma) [16]. Further chloroform extractions were done, an equal volume of cold absolute ethanol added, the DNA spooled out, dissolved in 3 ml SSC buffer and stored at 4°C over chloroform. The integrity of the high molecular weight DNA was checked by electrophoresis of 2.5 μg of each preparation at 2 $V \cdot cm^{-1}$ for 6 h in a 0.5% agarose gel containing 0.25 $mg \cdot l^{-1}$ ethidium bromide. DNA concentrations were estimated as described by Maniatis et al. [16], and 2.5 μg of each dot-blotted onto a nitrocellulose filter as described by Green et al. [17].

3.4. Hybridisation with the IS1 DNA probe

The specific IS1 DNA probe used was a 240-bp *Hae*III fragment from IS1-L (one of the IS1 copies flanking the chloramphenicol resistance transposon Tn*9*) in pJL3-1974 [18]. This fragment was cloned into the *Sma*I site of pUC8 [19] by blunt-end ligation with T4 ligase (Fig. 1). After digesting pUC8 with a combination of *Eco*RI and *Bam*HI, the fragment was purified by 2 PAGE runs (5% polyacrylamide and 4 $V \cdot cm^{-1}$ for 6 h). The frag-

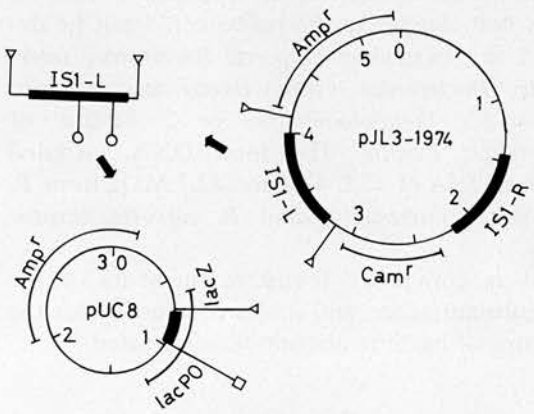

Fig. 1. Construction of specific IS1 DNA probe. A *Bam*HI (▽) fragment of pJL3-1974 containing IS1-L of Tn*9* was extracted and a 240-bp *Hae*III (○) fragment cloned into the *Sma*I site of pUC8. The 240-bp IS1 probe was extracted after digesting pUC8 with *Bam*HI and *Eco*R1 (□). Ampicillin resistance gene (Amp^r), chloramphenicol resistance gene (Cam^r), lactose operon genes (*lacOPZ*). Plasmid sizes shown in Kb.

ment was then end-labelled with [^{32}P]GTP (Amersham) using DNA polymerase I Klenow fragment [16]. Extraction of DNA fragments from gels, solutions for electrophoresis, solutions and conditions for all enzymes (BRL), hybridisation procedures and autoradiography were as described by Maniatis et al. [16].

3.5. Detection and sizing of plasmids

Small-scale plasmid preparations of 10-ml broth cultures were prepared as described by Maniatis et al. [16] and plasmids were sized using the method of Rochelle et al. [20].

4. RESULTS AND DISCUSSION

3 of the *Bacteroides* spp. *B. fragilis* NCTC9343, *B. intermedius* NCTC9336 and *B. corporis* ATCC33547, contained plasmid DNA of 30.2, 42.7 and 42.7 MDa, respectively. However, DNA homologous to the IS1 probe was not detected in total DNA from any of the *Bacteroides* spp. tested (Fig. 2). Although the probe did not include the whole of IS1, it was specific for IS1 DNA and covered 13 and 201 bp of the 2 conserved reading frames, *insA* and *insB,* respectively [5,6]. It can therefore be concluded that IS1 is not present in either chromosomal or plasmid DNA of the *Bacteroides* spp. tested.

Transfer of antibiotic resistance plasmids between various *Bacteroides* spp. [21,22] and between *Bacteroides* spp. and *E. coli* [23,24] has been demonstrated. Therefore, the dissemination of IS1 among intestinal *Bacteroides* spp. might be expected because of their close association with members of the Enterobacteriaceae. There are 2 possible explanations for the results obtained. *Bacteroides* spp. may have diverged from the Enterobacteriaceae before IS1 evolved and genetic transfer has not occurred between the two groups of bacteria. Alternatively, *Bacteroides* spp. and Enterobacteriaceae may be very distantly related and, although there is some genetic interchange between them, IS1 cannot function or be stably maintained in *Bacteriodes* spp. There is some evidence that the latter may be the case. Although the chondroitin lyase gene from *B. thetaiotaomicron* cloned into *E. coli* was expressed, the protein had a lower molecular weight [25]. This suggests that *E. coli* RNA polymerase may recognise different start and stop signals. Also, the transposon Tn*4400*, which codes for clindamycin resistance in *B. fragilis,* carries a tetracycline gene which is not expressed in *B. fragilis.* However, when cloned into *E. coli,* Tn*4400* can express tetracycline resistance [26]. It is therefore possible that *E. coli* genes are not readily expressed in distantly related *Bacteroides* spp and that IS1 could not function as a transposon.

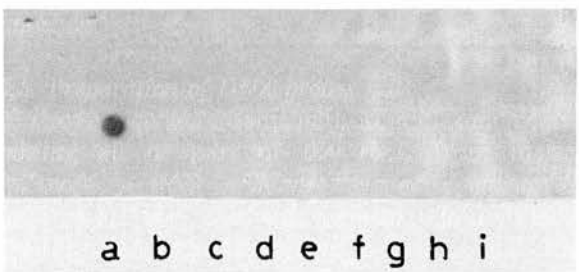

Fig. 2. Dot hybridisations of bacterial DNAs probed with IS1 DNA. *E. coli* K12 NCIB10083 (a), *B. intermedius* NCTC9336 (b), *B. corporis* ATCC33545 (c), *B. ovatus* ATCC8483 (d), *B. vulgatus* NCTC10583 (e), *B. thetaiotaomicron* NCTC10582 (f), *B. fragilis* NCTC9343 (g), *B. fragilis* JC19 (h) and *P. aeruginosa* 8602 (i).

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Immunochemical Characterization of a Polysaccharide Antigen of *Bacteroides fragilis* with an IgM Monoclonal Antibody

By JOHN H. REID,[1]*† SHEILA PATRICK[2] AND SOAD TABAQCHALI[1]

[1] *Department of Medical Microbiology, St Bartholomew's Hospital Medical College, London EC1A 7BE, UK*

[2] *Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK*

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An IgM mouse monoclonal antibody (McAb) Bf4 was produced to a surface polysaccharide of *Bacteroides fragilis* NCTC 9343. Immunoblotting showed that McAb Bf4 reacted strongly with a high molecular mass structure which was sensitive to oxidation with periodate but resisted protease treatment. An inhibition enzyme-linked immunosorbent assay (ELISA) indicated that McAb Bf4 did not cross react with the sixteen *Bacteroides* species and strains tested. Cells of *B. fragilis* NCTC 9343 recovered from the various interfaces of a Percoll discontinuous density gradient were tested in the inhibition ELISA. Bacteria from the 0–20%, 20–40% and 40–60% interfaces inhibited the ELISA; however, cells from the 60–80% interface did not. Electron microscopy with immunogold labelling showed that McAb Bf4 did not react with the extracellular fibrous network on bacteria recovered from the 0–20% interface, or the extracellular electron dense layer on cells from the 60–80% interface; however, it was associated with a surface structure on cells from the 20–40% interface. Growth *in vivo* did not enrich for bacteria with this structure.

INTRODUCTION

Bacteroides fragilis is the most common Gram-negative anaerobic organism isolated from clinical specimens (Gorbach & Bartlett, 1974; Duerden, 1980). However, the factors responsible for the enhanced virulence of this species are unknown despite the development of a number of models for studying the pathogenic mechanisms of bacteroides (Hofstad, 1984). Previous reports have suggested that the capsular polysaccharide of *B. fragilis* may represent a virulence factor (Onderdonk *et al.*, 1977; Connolly *et al.*, 1984).

A number of *B. fragilis* surface antigens have been identified (Kasper, 1976; Babb & Cummins, 1978; Cousland & Poxton, 1983; Lambe *et al.*, 1984; Weintraub *et al.*, 1985), but little is known about the distribution and expression of these structures. Variation in the degree of capsulation of *B. fragilis* occurs during passage *in vitro* and *in vivo* (Kasper *et al.*, 1980; Patrick & Reid, 1983; Patrick *et al.*, 1984). Cells of *B. fragilis* with different sizes of capsule can be separated on a Percoll density gradient and appear to differ structurally when examined by electron microscopy (Patrick *et al.*, 1986). The relationship between the surface structures observed by us and either the surface polysaccharide described as 'capsule' by Kasper (1976) or the glycocalyx reported by Lambe *et al.* (1984) is unknown. Characterization of these antigens using highly specific monoclonal antibodies (McAbs) and immunochemical techniques, e.g. immunoblotting and electron microscopy with immunogold labelling, will provide a way of comparing the surface structures identified by different research groups. McAbs have been produced to rough LPS molecules of *B. fragilis* (Linko-Kettunen *et al.*, 1984) and a high molecular mass surface structure on *B. fragilis* (Reid *et al.*, 1985).

† Present address: Coralab Research, Huntingdon Road Laboratories, Cambridge CB3 0DJ, UK.

Abbreviations: McAb, monoclonal antibody; TBS, Tris-buffered saline; CB, cacodylate/HCl buffer; PCP, phenol–chloroform–petroleum.

In this study immunoblotting was used to define the immunochemical nature of the structure recognized by McAb Bf4 and an inhibition enzyme-linked immunosorbent assay (ELISA) was used to determine the specificity for *Bacteroides* strains and species. The distribution of the Bf4 epitope on *B. fragilis* populations grown *in vitro* and *in vivo* was investigated using immunofluorescence and electron microscopy with immunogold labelling.

METHODS

Bacterial growth conditions. The *Bacteroides* strains used in this study were as follows: *B. fragilis* NCTC 9343 from the Department of Microbiology and Immunobiology, the Queen's University of Belfast, UK; *B. fragilis* GNAB 4 from the Department of Bacteriology, Edinburgh University Medical School, UK; *B. ovatus* ATCC 8483, *B. distasonis* ATCC 8503, *B. thetaiotaomicron* NCTC 10582, *B. fragilis* ATCC 23745, NCTC 9344, NCTC 10584, NCTC 8560 and clinical *B. fragilis* isolates Bf12, Bf17, Bf18, Bf19, Bf20, Bf21, Bf25 and Bf30 from the Department of Medical Microbiology, St Bartholomew's Hospital Medical College, London, UK. *B. fragilis* strains grown to late exponential phase in defined broth (van Tassell & Wilkins, 1978) or on horse blood agar plates were incubated at 37 °C in an anaerobic chamber (Forma) with an atmosphere of 80% (v/v) N_2, 10% (v/v) H_2 and 10% (v/v) CO_2. Identification was confirmed using the API system.

Production of McAbs. A BALB/c mouse was immunized with whole cells of *B. fragilis* NCTC 9343. The mouse was inoculated intraperitoneally each week for 3 weeks with 0·2 ml of a bacterial suspension of $1{\cdot}0 \times 10^8$ c.f.u. in one-quarter strength Ringer's solution, and an additional inoculation of 0·2 ml was given 4 d before fusion. Spleen cells from the immunized mouse were fused with x 63 Ag-8.653 myeloma cells using the polyethylene glycol fusion technique (Lemke *et al.*, 1978). Hybrid cell lines were selected in RPMI 1640 medium containing hypoxanthine–aminopterin–thymidine and 15% (v/v) foetal calf serum (Gibco). Hybridoma culture supernate was screened for antibody to *B. fragilis* NCTC 9343 by ELISA (see below). Hybridoma culture supernate was used in all experiments.

The hybridoma cell line Bf4 was cloned using a limiting dilution method and BALB/c macrophages were used to assist the growth of hybridoma clones. Cells were concentrated by centrifugation and 1 ml samples stored at −80 °C in growth medium containing 15% (v/v) foetal calf serum and 10% (v/v) dimethyl sulphoxide. Hybridoma cells from this stock were used to produce large quantities of hybridoma Bf4 culture supernate.

ELISA. An indirect ELISA was used to screen for McAb producing hybridoma cell lines. Wells of PVC microtitre plates (Becton and Dickenson) were each coated with bacteria (1×10^6 cells ml^{-1}) suspended in 50 mM-sodium carbonate buffer, pH 9·6. The plates were incubated at 37 °C for 2 h and washed three times with 50 mM-sodium phosphate buffer, pH 7·4, containing 150 mM-NaCl (PBS) and 0·05% (v/v) Tween 20. Plates were stored at −20 °C.

Hybridoma culture supernate (100 µl) was added to each well, and the plates were incubated at 37 °C for 1 h and washed as before [dilutions, if required were made in PBS containing 1% (w/v) bovine serum albumin (BSA–PBS)]. Goat anti-mouse IgM (Mu chain specific) and IgG-alkaline phosphatase conjugates (Tago) diluted 1 in 3000 in 1% (w/v) BSA–PBS were added to each well (100 µl) and the plates were incubated at 37 °C for 1 h. After washing, 100 µl of *p*-nitrophenyl phosphate (Sigma) (1 mg ml^{-1}) in 50 mM-sodium carbonate buffer, pH 9·8, containing 1 mM-$MgCl_2$ was added to each well. The plate was incubated at 37 °C for 1 h; the reaction was stopped with 100 µl 3 M-NaOH and absorbance (at 405 nm) was read using a BioRad automatic plate reader.

An inhibition ELISA was used to test for cross reactivity with other bacteroides. Bacteria suspended in 0·5 ml 2% (w/v) BSA in PBS to a concentration of $5{\cdot}0 \times 10^8$ c.f.u. ml^{-1} were mixed with 0·5 ml of hybridoma culture supernate containing McAb Bf4 and incubated at 4 °C for 18 h. After centrifugation at 5000 ***g***, triplicate samples (100 µl) of the supernate were tested on ELISA plates coated with *B. fragilis* NCTC 9343.

An ELISA was used to determine the immunoglobulin class of McAb Bf4. Sheep anti-mouse IgG2a (Serotec), goat anti-mouse IgG1 (Sigma), IgG2b (Sigma), IgG3 (Sigma) and IgM (Sigma) were diluted in 50 mM-sodium carbonate buffer, pH 9·6, and coated onto PVC microtitre plates as before. Hybridoma culture supernate (50 µl) was added to each well; the plates were incubated for 2 h at 37 °C, and then washed as before. Goat anti-mouse IgG (specific for light and heavy chains) conjugated to horse-radish peroxidase (Sigma) was diluted 1 in 1000 in 1% (w/v) BSA in PBS and 50 µl was added to each well. The plates were incubated at 37 °C for 1 h and washed as above. Orthophenylene diamine (Sigma) substrate (40 mg) was dissolved in 100 ml of a solution containing 100 mM-citric acid, 200 mM-Na_2HPO_4 and 0·01% (w/v) H_2O_2; 50 µl of the solution was added to each well and the plates were incubated for 30 min; the reaction was stopped with 1 M-H_2SO_4 and the absorbance read at 520 nm.

Enrichment for bacterial variants. Capsulate and non-capsulate *B. fragilis* (defined by light microscopy) were separated by Percoll density gradient centrifugation (Patrick & Reid, 1983).

Preparation of antigens. Antigens were extracted from bacteria by the aqueous phenol method of Westphal & Luderitz (1954). The aqueous phase was centrifuged at 10000 ***g*** for 30 min at 4 °C to remove insoluble material and the supernate was stored at −20 °C.

Antigens from bacteria were also extracted with a glycine buffer containing EDTA. Bacteria harvested from

500 ml broth were resuspended in 10 ml 3 mM-glycine buffer, pH 5·0, containing 10 mM-EDTA, and were incubated at 37 °C for 30 min. Whole cells were removed by centrifugation at 10000 *g* for 30 min at 4 °C and the extract was stored at −20 °C. Extracts were treated with protease or sodium periodate by the method of Cousland & Poxton (1984).

Carbohydrate assay. The anthrone method of Morris (1948), with D-glucose as a standard, was used.

SDS-PAGE. This was done on 10% vertical slab gels using the Laemmli buffer system (Laemmli, 1970) at a constant current of 40 mA per gel.

Immunoblotting and enzyme immunoassay. The method of Towbin *et al.* (1979) was used with the following modifications. Material was transferred to a nitrocellulose membrane (Transblot TM transfer medium; BioRad) in 25 mM-Tris/192 mM-glycine buffer, pH 8·3, containing 20% (v/v) methanol at 350 mA for 1·5 h. After washing for 10 min in Tris-buffered saline (TBS; 20 mM-Tris/HCl, 500 mM-NaCl, pH 7·5) the membrane strip containing the protein molecular mass standards was fixed with 1% (v/v) acetic acid and stained with amido black. The rest of the membrane was placed in 3% (w/v) BSA in TBS (BSA–TBS) for 16 h at 4 °C. It was then transferred into hybridoma culture supernate, or an appropriately diluted mouse derived antiserum, and incubated for 2 h at room temperature. After three washes in 0·025% (v/v) Tween 20 in TBS, the membrane was placed in goat anti-mouse IgM-alkaline phosphatase conjugate (Tago) specific for the Mu chain, then diluted 1 in 3000 in 1% (w/v) BSA in TBS and incubated for 1 h at room temperature. The membrane was washed three times in Tween 20–TBS and placed in 50 mM-Tris/HCl buffer, pH 8·2, containing Fast Red (3 mg ml^{-1}) and naphthol-AS-MX-phosphate (0·2 mg ml^{-1}). Colour development took place in 5–15 min, and the reaction was then stopped by washing in distilled water. All the above steps were done with gentle agitation throughout.

Immunogold labelling and electron microscopy. Bacteria were washed twice in 100 mM-sodium cacodylate/HCl buffer, pH 7·2 (CB), and fixed in CB containing 2% (v/v) paraformaldehyde and 0·1% (v/v) glutaraldehyde for 1 h at 4 °C. The cells were again washed in CB, dehydrated in graded alcohols and embedded in LR white resin. For gold labelling, ultrathin sections on nickel grids were treated with McAb Bf4 followed by goat anti-mouse IgM–40 nm gold conjugate (Janssen). The grids were washed in 0·1% (v/v) BSA–TBS, pH 8·2, and rinsed in distilled water. They were then stained with uranyl acetate and lead citrate and examined with a Philips 301 transmission electron microscope. Ruthenium red staining was done as described previously (Patrick *et al.*, 1986) before the bacteria were embedded in LR white resin.

Immunofluorescence microscopy. The method was described by Reid *et al.* (1985).

RESULTS

SDS-PAGE and immunoblotting of the phenol–water extract

Reactions with polyclonal mouse antiserum. SDS-PAGE and immunoblotting with homologous anti-*B. fragilis* NCTC 9343 antiserum detected multiple bands (Fig. 1*c*). A diffuse staining pattern and a series of closely spaced discrete bands (arrowed S) were detected at the top of the blot. A series of regularly spaced discrete bands was observed in the middle of the blot (labelled R+n). At least two heavily stained bands were seen in the lower region of the blot.

Reaction with McAb Bf4. Staining was observed only in the high molecular mass region when McAb Bf4 was used for immunoblotting (Fig. 1*b*). McAb Bf4 reacted strongly with both the diffuse staining region and the closely spaced discrete bands.

Treatment of antigen with protease, periodate and heat. The structure recognized by McAb Bf4 resisted protease digestion (result not shown) and the immunoblot was identical to that for the control antigen (Fig. 1*b*). Oxidation of the antigen preparation with sodium periodate destroyed the Bf4 antigenic determinant (Fig. 1*a*). Heating at 100 °C for 3 min did not destroy the structure containing the Bf4 epitope.

SDS-PAGE and immunoblotting of glycine–EDTA extract

Immunoblotting of the glycine–EDTA extract after SDS-PAGE with McAb Bf4 revealed multiple closely spaced discrete bands and at least four extra bands in the high molecular mass region of the blot (Fig. 2*b, d*). Protease treatment did not affect the closely stained bands but the extra bands in the top region of the blot disappeared (Fig. 2*c*). Oxidation with sodium periodate removed the closely spaced bands, but multiple faintly immunoreactive bands were detected in the lower region of the blot after treatment (Fig. 2*a*).

ELISA

McAb Bf4 was identified as an IgM antibody using a sandwich ELISA.

Inhibition studies were done to test the reactivity of McAb Bf4 with other *Bacteroides* species

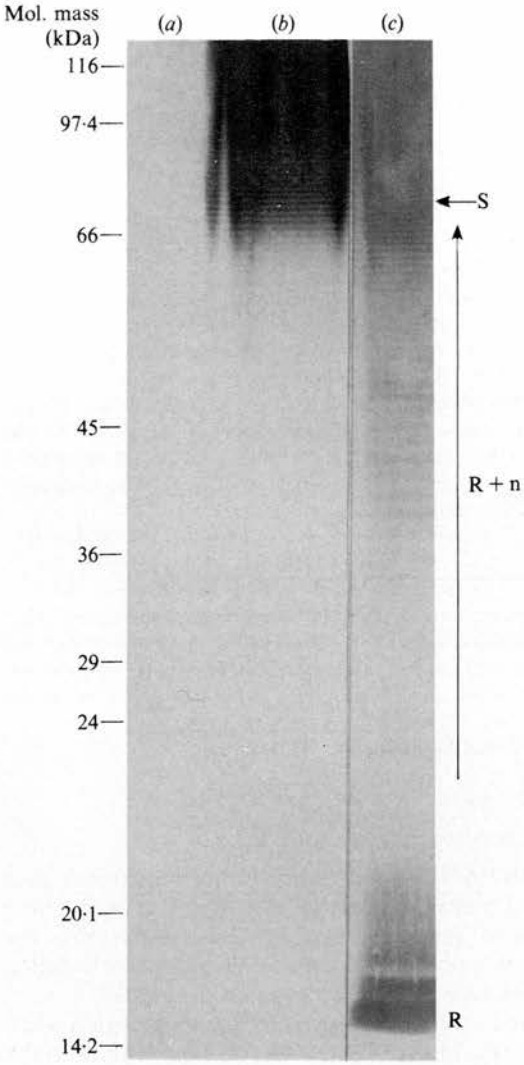

Fig. 1. SDS-PAGE and immunoblot analysis of aqueous phase antigens extracted from *B. fragilis* NCTC 9343 by the hot phenol–water method. Periodate treated antigen (*a*) and control (*b*) were blotted with McAb Bf4; control antigen was also blotted with immune mouse antiserum (*c*). Carbohydrate loaded was 15 μg per lane.

and strains. Also, cells of *B. fragilis* NCTC 9343 recovered from the various interfaces of a Percoll discontinuous density gradient were tested for expression of the Bf4 antigen.

B. ovatus ATCC 8483, *B. distasonis* ATCC 8503 and *B. thetaiotaomicron* NCTC 10582 did not inhibit the ELISA. Four reference strains and nine clinical isolates of *B. fragilis* were also tested. Inhibition was detected only with the homologous strain of NCTC 9343.

Bacteria recovered from the 0–20%, 20–40% and 40–60% interfaces of a Percoll discontinuous density gradient inhibited the ELISA, but cells from the 60–80% interface did not. No attempt was made to measure the minimum number of cells required to inhibit the assay.

Immunofluorescence and immunoelectron microscopy

Immunofluorescence and immunoelectron microscopy of cells taken from the four interfaces of the Percoll gradient gave the same pattern of labelling. The absence of the Bf4 epitope from the 60–80% interface was confirmed. Cells from this interface are apparently non-capsulate by light microscopy, but have an extracellular electron dense layer by electron microscopy (Patrick

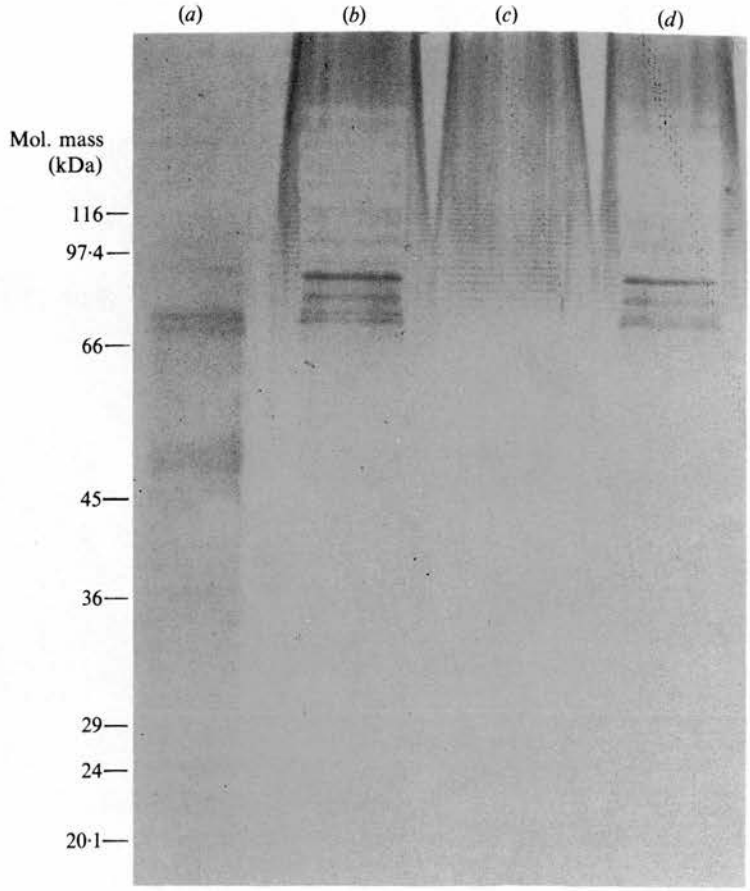

Fig. 2. SDS-PAGE and immunoblot analysis of antigens extracted from *B. fragilis* NCTC 9343 with mild heat and EDTA–glycine buffer, pH 5·0. Antigen treatments were periodate (*a*) with control (*b*), and protease (*c*) with control (*d*). Carbohydrate loaded was 50 μg per lane.

et al., 1986). A low proportion of cells from the 0–20% (Fig. 3*a*) and 40–60% (not illustrated) interfaces were labelled, but cells from the 20–40% interface were enriched for the Bf4 epitope (Fig. 3*b*). The surface structures of *B. fragilis* are not easily resolved with the fixation procedures required to preserve the reactivity of the antigens for immunoelectron microscopy: therefore duplicate samples were stained with ruthenium red. The results indicate that the Bf4 epitope is associated with the marginal fibrous network present on cells from the 20–40% interface (Fig. 3*d*), but not with the extensive fibrous network which is present on cells from the 0–20% interface (Fig. 3*c*). Two other monoclonal antibodies (Bf1; Reid *et al.*, 1985 and Bf2; not illustrated) give a similar labelling pattern for each of the interfaces and could not be related to a specific surface structure.

Cells from the 0–20% interface were grown for 24 h in chambers implanted in the mouse peritoneal cavity (Patrick *et al.*, 1984). These populations were not enriched for the Bf4 epitope (Fig. 4). Similar labelling patterns were seen with these populations before and after *in vivo* passage (Figs 3*a* and 4).

DISCUSSION

Immunoblotting and immunoelectron microscopy showed that McAb Bf4 reacted with an epitope on a high molecular mass surface polysaccharide. This structure was distinct from the

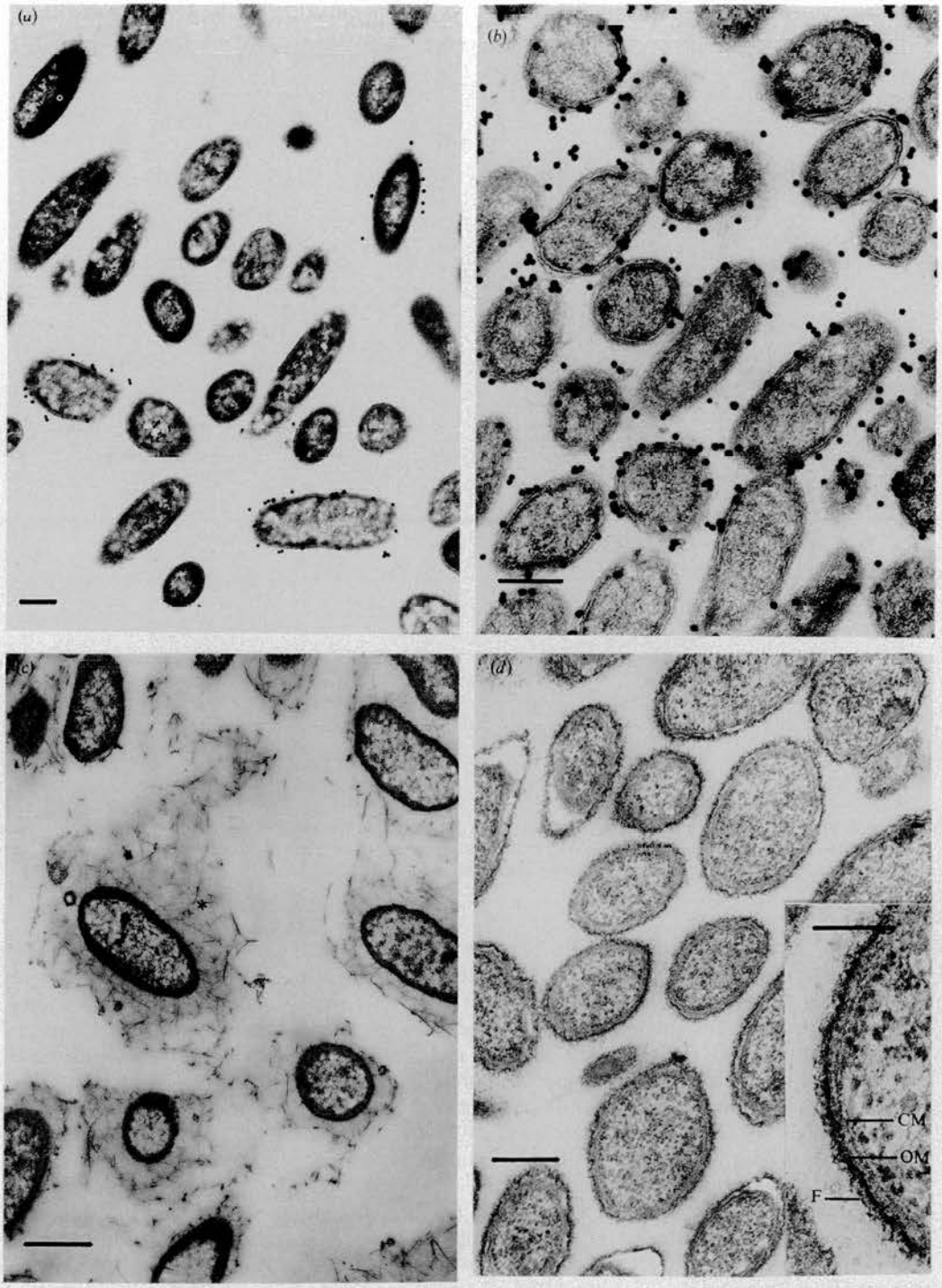

Fig. 3. Electron micrographs of cells of *B. fragilis* NCTC 9343 recovered from the interfaces of a Percoll discontinuous density gradient. (*a*) Cells from the 0–20% interface labelled with McAb Bf4 and anti-mouse IgM immunogold conjugate; note the low proportion of gold labelled cells. (*b*) Cells from the 20–40% interface labelled as in (*a*); note that most of the cells are labelled. (*c*) Cells from the 0–20% interface stained with ruthenium red; note the extensive fibrous network (*). (*d*) Cells from the 20–40% interface stained with ruthenium red; note the marginal fibrous network (F) adjacent to the outer membrane (OM); CM, cytoplasmic membrane. Bar = 0·5 μm in main micrographs and 100 nm in Fig. 3(*d*) insert.

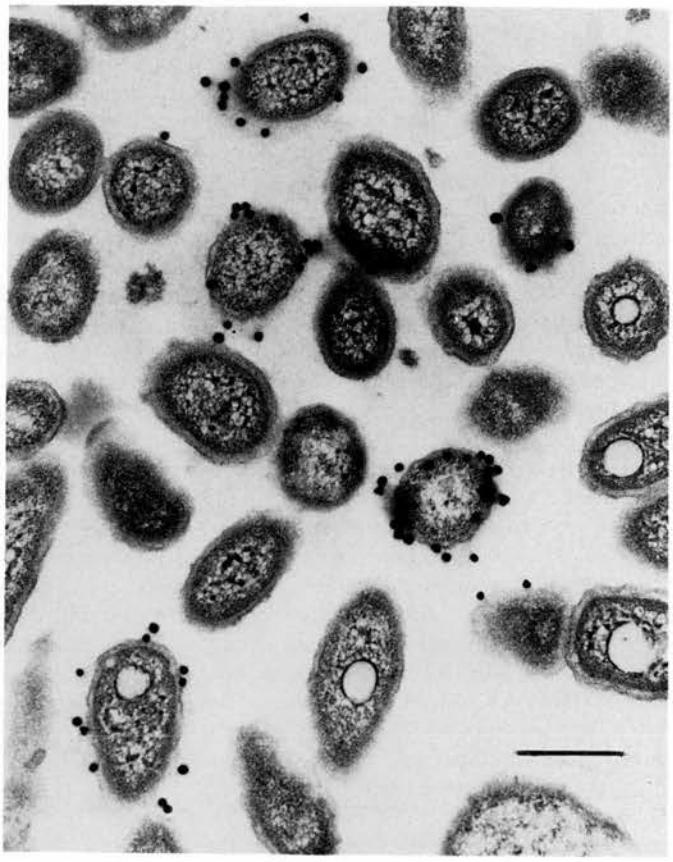

Fig. 4. Electron micrograph of cells of *B. fragilis* NCTC 9343 taken from the 0–20% interface of a Percoll gradient and passaged for 24 h in the mouse peritoneal cavity. Cells were labelled with McAb Bf4 and anti-mouse IgM immunogold conjugate. Note the low proportion of cells labelled. Bar = 0·5 μm.

extensive fibrous network, present on cells recovered from the 0–20% interface of a Percoll density gradient, and the electron dense layer, present on cells from the 60–80% interface. It was, however, associated with the marginal fibrous network observed on cells from the 20–40% interface. These results indicate that structures which look different by electron microscopy may also be antigenically different. Immunogold labelling of cells grown in the mouse peritoneal cavity gave a heterogeneous labelling pattern which indicates that this epitope was not selected *in vivo*.

ELISA inhibition studies showed that McAb Bf4 recognized a type-specific surface antigen. Kasper *et al.* (1983) showed that extracted capsular polysaccharide from *B. fragilis* NCTC 9343 was chemically and antigenically different from that of *B. fragilis* ATCC 23745, and type-specific epitopes were identified in the capsular material. This capsular material was detected in the high molecular mass region of a 20% (w/v) acrylamide gel by silver staining (Weintraub *et al.*, 1985). Weintraub *et al.* (1985) also extracted LPS from *B. fragilis* NCTC 9343 by the hot phenol–water method and subsequently extracted the aqueous phase with a phenol–chloroform–petroleum (PCP) mixture: rough LPS only was detected with SDS-PAGE and silver staining. However, *B. fragilis* NCTC 9343 could also contain smooth LPS with long polysaccharide side chains since the PCP extraction procedure preferentially extracts lipophilic rough LPS while smooth LPS is excluded (Galanos *et al.*, 1969). Cousland & Poxton (1983) have observed heterogeneity in LPS extracted from a variety of *B. fragilis* strains by the hot phenol–water method. They separated LPS extracted from *B. fragilis* GNAB 92 by electrophoresis on a 10% (w/v) acrylamide gel and detected possible smooth LPS (labelled S) in the top of the blot.

McAb Bf4 reacted with *B. fragilis* NCTC 9343 antigens in this region of the blot. McAb Bf4 did not react with the typical LPS 'ladder' (Fig. 1*b*, labelled R + n) in the middle of the blot, suggesting that either the Bf4 epitope was not part of a repeating O-side-chain or the concentration of the LPS containing short O-side-chains was below a detectable level when using McAb Bf4. The epitope could be associated with a repeating capsular polysaccharide unit but the polymer would need to be covalently linked to a lipid to bind SDS, or have a negative charge for electrophoresis to occur.

E. coli LPS formed non-covalently linked multimers during SDS-PAGE when 0·1% (w/v) SDS was used in 15% (w/v) acrylamide separating gels (Peterson & McGroarty, 1985). The multimers dissociated when 0·5% (w/v) SDS was used or when separating gels were overlayed with butanol and left overnight. McAb Bf4 could also be reacting with multimers of *B. fragilis* NCTC 9343 LPS. The SDS-PAGE conditions were not altered in this study but the hypothesis could be tested by increasing the SDS concentrations in both the sample buffer and the separating gel, or by electro-eluting the high molecular mass material from the top section of the gel, and re-analysing the material by SDS-PAGE and immunoblotting with McAb Bf4.

Poxton *et al.* (1985) detected protein–LPS complexes when outer membrane extracts of *Pseudomonas aeruginosa* were immunoblotted with McAbs specific for the LPS O-antigen. This report describes a similar observation using a glycine–EDTA extract of *B. fragilis* NCTC 9343 and immunoblotting with McAb Bf4. There are a number of possible explanations for this phenomenon. First, protein–LPS complexes released by the glycine–EDTA treatment could resist solubilization with SDS. Second, antigens could be completely solubilized by detergent but co-migrate to the same position in the gel. Third, solubilized high molecular mass peptides could become entangled with long polysaccharide chains containing the Bf4 epitope during electrophoresis. The stability of antigen complexes could be investigated by affinity chromatography. We now hope to purify the polysaccharide containing the Bf4 epitope using affinity chromatography. The structure could then be chemically analysed to determine whether it is a polysaccharide or a lipopolysaccharide.

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4

Phagocytosis of *Bacteroides fragilis in vitro*

Sheila Patrick
Department of Microbiology and Immunobiology,
The Queen's University of Belfast,
Belfast, UK

1. Abstract

Although *Bacteroides fragilis* is the Gram-negative anaerobic bacterium most commonly isolated from clinical infections the nature of the surface antigens involved in its virulence remains unclear. The homogeneity of surface antigens within populations of *B. fragilis* has been assumed in much of the published literature; however, electron microscopy and labelling with monoclonal antibodies have revealed the heterogeneity of surface structures and antigens within populations of individual strains of *B. fragilis*. Three distinct extracellular structures of different size can be observed with the electron microscope; namely a large capsule, a small capsule and an electron dense layer. Bacteria with these structures can be separated by Percoll density gradient centrifugation. Subsequent culture enriches for populations carrying these structures. In-vitro studies of phagocytosis of *B. fragilis* indicate that the large capsule impedes phagocytic uptake where it is present in sufficient quantity; however, populations of *B. fragilis* NCTC 9343 growing in the mouse peritoneal cavity rapidly lose the ability to produce this structure. This occurs both in the presence and in the absence of phagocytes. Labelling with a monoclonal antibody specific for the small capsule has shown that (a) the population does not switch from production of the large capsule to production of the small capsule and (b) that cells with the small capsule are phagocytosed *in vivo*. Labelling with a number of different monoclonal antibodies showed that populations growing *in vivo* were antigenically heterogeneous.

2. Introduction

Bacteroides fragilis is a frequent isolate from such infections as bacteraemia, wound, intra-abdominal and urogenital infection (1). As yet, the factors that enable it to be a successful pathogen are not clearly understood. Extracellular encapsulating structures have long been associated with the virulence of bacteria (2) where they may act as a barrier to phagocytosis. Many investigations of *B. fragilis* pathogenesis have centred on the possible role of similar structures in its virulence (3).

A crude extract of extracellular polysaccharides from *B. fragilis* strain ATCC 23745 produced abscesses in a rat model of infection (4). Immunization of both rats (5) and mice (6) with the crude extract protected against abscess formation by this strain. The immunity was apparently T-cell mediated.

Pathogenic synergy between bacteroides and enterobacteria occurs in natural infections. This may be related to the inhibitory effect of bacteroides on the phagocytosis of other genera of bacteria. A number of researchers, e.g. Jones and Gemmel (7) and Wel *et al.* (8), have examined the interaction of *Bacteroides* spp. with other bacteria in in-vitro studies of phagocytic uptake and killing. Undoubtedly the presence of *Bacteroides* spp. can diminish the phagocytic uptake and killing of other bacterial species, probably because of the depletion of opsonins they cause. This may be associated with some factor present in crude extracts of the surface polysaccharides (9). The precise mechanism of the protection afforded by *Bacteroides* spp. remains unknown.

A more detailed analysis of the surface carbohydrates of *B. fragilis* has shown that aqueous phenol extractions of polysaccharide contain a complex mixture of antigens. Most strains of *B. fragilis* have four different antigens, namely a rough lipopolysaccharide (LPS), smooth LPS, a common carbohydrate antigen and a high molecular weight polysaccharide (10).

The presence of fimbriae on some stains of *B. fragilis* has been associated with susceptibility to phagocytosis and the ability to agglutinate erythrocytes (11). It has also been suggested that capsules (12) and surface polysaccharides (13) may be involved in haemagglutination.

Unfortunately in all the above studies qualitative and quantitative differences in the surface structures and antigens *within* the populations examined were largely unknown.

3. Definition of Surface Structures

A range of extracellular capsule sizes has been observed in wet India ink-stained preparations of *B. fragilis* isolates examined by light microscopy (14). Size differences occurred both within and between strains. Cells from *B. fragilis* cultures can be separated according to the size of these surface structures by Percoll step density gradient centrifugation (15). Bacteria with large capsules

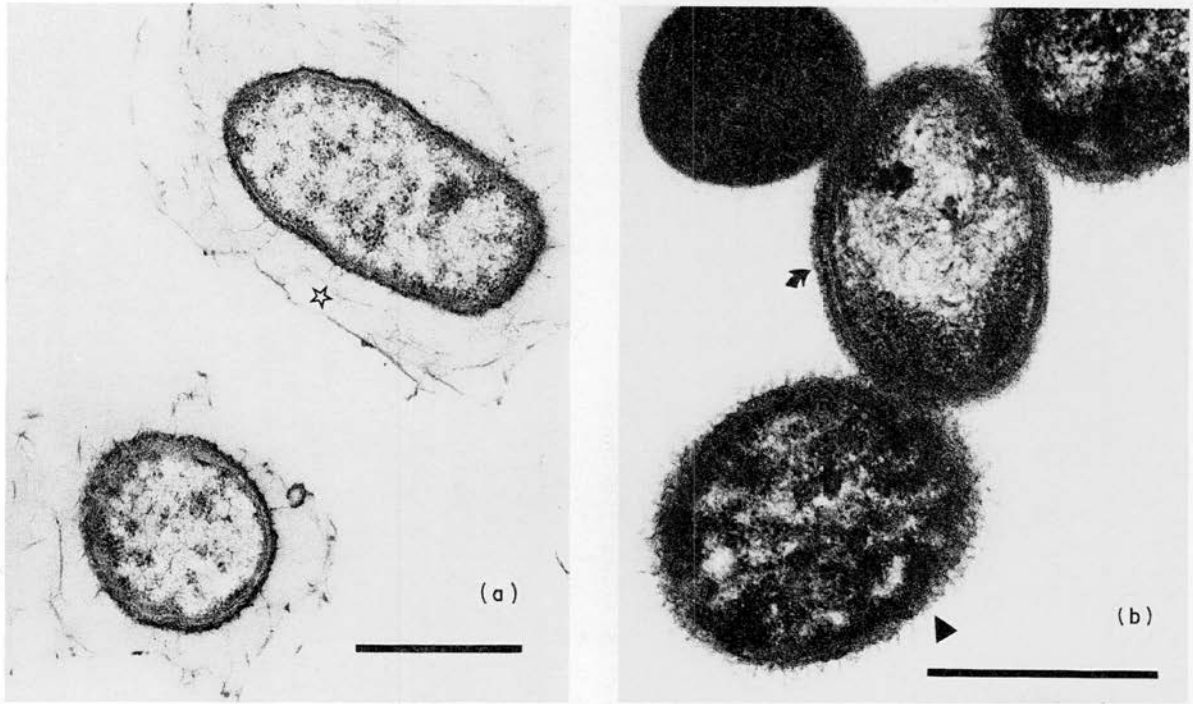


Figure 1. Electron micrographs of *B. fragilis* NCTC 9343 grown in a defined broth. (a) Note the large capsule (star). (b) Note the small capsule (straight arrow) and the electron dense layer (curved arrow). Scale bar in all figures = 0.5 μm.

remain on top of the gradient and those with smaller capsules are found further down the gradient. If bacteria are taken from the Percoll gradient interface layers and subcultured there is some enrichment for the different sizes of capsules. Therefore production of different sizes of capsules is a relatively stable trait in in-vitro subculture.

Cells with the following extracellular structures can be seen within one strain by electron microscopy (16):

1. bacteria with a large fibrous network, corresponding to a large capsule visible by light microscopy;
2. bacteria with a smaller fibrous network, corresponding to a small capsule visible by light microscopy; and
3. bacteria with a marginal electron dense layer which appear to be non-capsulate by light microscopy.

These three structures are illustrated in Figure 1 and will be referred to as the large capsule, small capsule and electron dense layer (EDL) respectively. A monoclonal antibody (McAb) has been produced which reacts specifically with an epitope associated with the small capsule (17). This indicates that the small capsule is antigenically different from the EDL. The proportion of cells with these different structures can vary with the strain, nutrient availability and the composition of the culture inoculum.

4. Surface Structures as Virulence Determinants: In-vitro Studies

Studies with populations homogeneous for the large capsule indicate that when bacteria with this structure are present in sufficient quantity, in relation to the numbers of human polymorphonuclear leukocytes (PMNL) present, phagocytosis is impeded. It is probable that the large capsule mops up opsonin present in normal human serum (18). Bacteria with the large capsule survive better under aerobic conditions than cells with just the EDL (19). This has implications in the initial stages of infections that result from faecal contamination. In such cases enhanced tolerance of oxygen would be of advantage to *B. fragilis*.

Cells with either a large or a small capsule do not haemagglutinate erythrocytes, whereas cells with the EDL haemagglutinate erythrocytes from a variety of mammalian species (20). The EDL may also be associated with resistance to killing by normal human serum (18).

5. Phagocytosis *in vivo*

5.1. Selection of populations

Intra-abdominal abscesses are commonly caused by *B. fragilis* (1) and the growth and survival of *B. fragilis* in chambers implanted in the mouse peritoneal

cavity has been studied (19). The growth of *B. fragilis* NCTC 9343, initially homogeneous for either the large capsule or the EDL, was examined in either the presence or the absence of leukocytes. The commonest phagocytes found inside the chambers were PMNL at a concentration of approximately 10^{6-7}/ml.

Examination of populations initially homogeneous for the large capsule indicated that the large capsule was not produced *in vivo* in either the presence or absence of phagocytes (Figure 2). Neither was there any selection of bacteria producing the large discrete capsule from populations initially homogeneous for the EDL. There are a number of possible explanations for this phenomenon. Firstly, the nutritional conditions *in vivo* may be such that the bacteria cannot produce the large capsule. Secondly, the bacteria may no longer be producing a discrete capsule, but releasing free slime. Finally there may be some selective disadvantage in production of a large capsule.

To determine if the in-vivo grown cells were still capable of producing the large capsule they were subcultured once *in vitro* and the proportion of cells

Figure 2. Electron micrographs of *B. fragilis* NCTC 9343, initially bearing the large capsule, after 24 h growth in chambers implanted in the mouse peritoneal cavity (*in vivo*). Note that the large capsule is now absent.

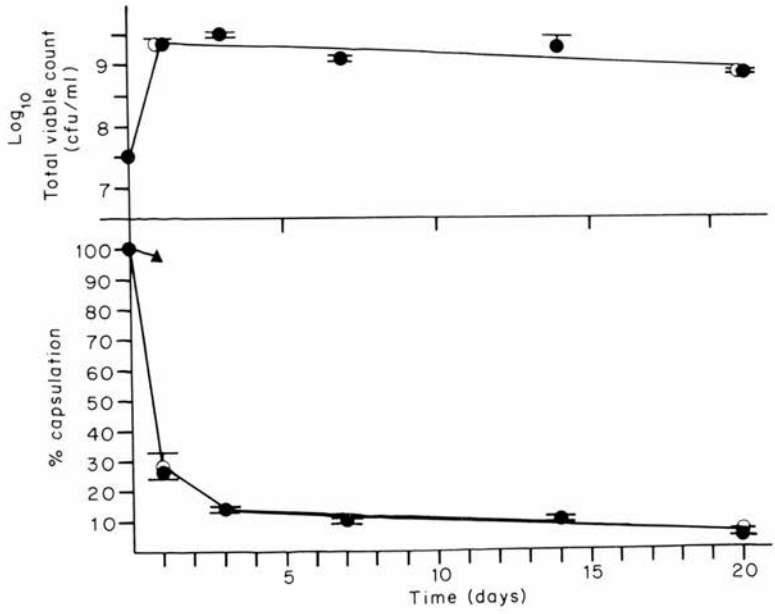


Figure 3. Total viable count and the proportion of *B. fragilis* NCTC 9343 that produces the large capsule (percentage capsulation) on subculture in defined broth: (●) after growth *in vivo* in the presence of leukocytes; (○) after growth *in vivo* in the absence of leukocytes; (▲) without growth *in vivo*. Note that the percentage capsulation after only in-vitro subculture remains high.

with the large capsule recorded by examination with the light microscope (Figure 3). Bacteria grown overnight in a broth culture of defined medium continue to produce the large capsule; whereas, after 24 h growth *in vivo*, there is a large decrease in the proportion of bacteria capable of producing the large capsule. There was little difference in this result whether phagocytes were present or absent. The total viable bacterial count (Figure 3) remained high during a 20-day period. This indicates that the phagocytes are having little impact on the viable numbers of bacteria, although phagocytosis was observed by electron microscopy (Figure 4).

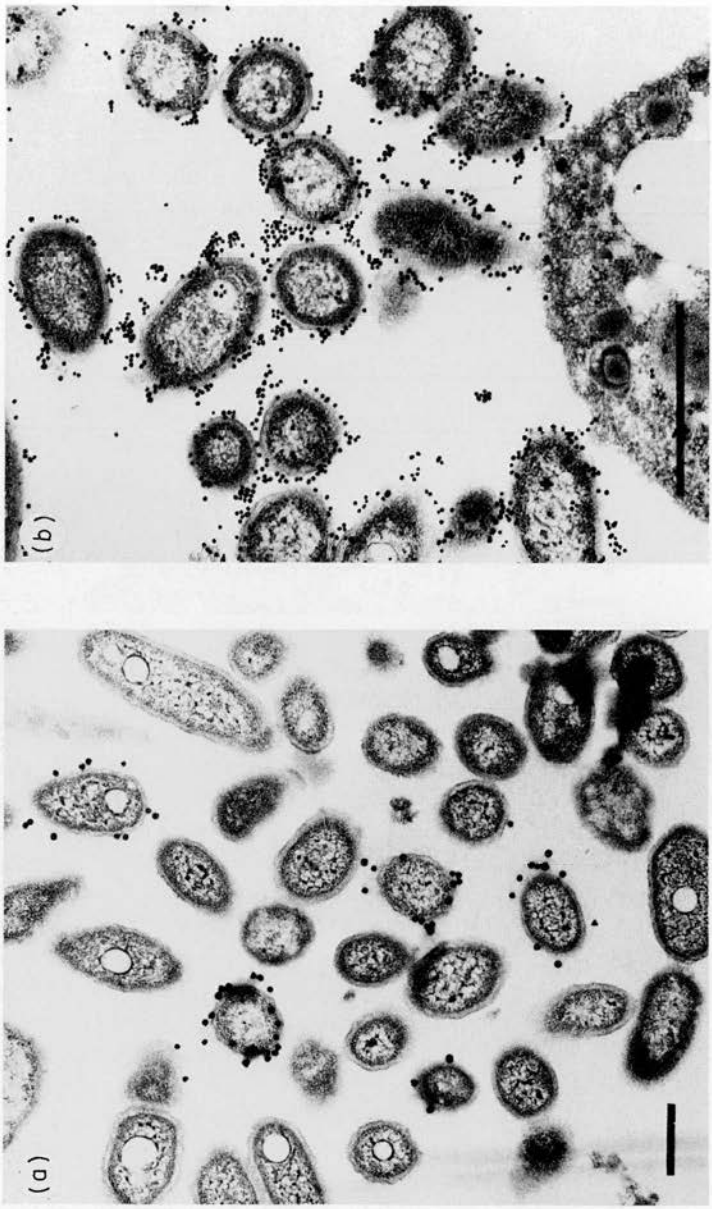
5.2. Surface antigens

Structures similar in appearance to the EDL and the small capsule have been observed on bacteria grown *in vivo*; however, the normal staining procedures for electron microscopy do not distinguish between material of bacterial origin and that of mouse origin. Mouse material could coat the bacterial cell surface.

Figure 4. Electron micrographs of *B. fragilis* NCTC 9343, initially bearing the large capsule, after 24 h growth *in vivo*. Note the phagocytosed bacteria.

Monoclonal antibodies provide specific probes which, coupled with immunogold labelling, can identify known antigens. Unfortunately the fixation procedures required to maintain the reactivity of the surface antigens result in the loss of detail of the structures under the electron microscope. Therefore, in order to characterize McAb, parallel samples must be fixed in the normal manner.

Bacteria that all expressed the large capsule before growth *in vivo* were labelled with McAb Bf4 which is specific for an epitope associated with the small capsule of *B. fragilis* NCTC 9343 (for details of characterization see Chapter 17). A proportion of the population will bind the McAb (Figure 5a), but there is no obvious enrichment for bacteria expressing this epitope, even after a 20-day period in the mouse peritoneal cavity. The heterogeneous labelling pattern was not caused by the washing procedures required for labelling as all the bacteria in populations homogeneous for the small capsule were labelled, as were in-vivo grown bacteria labelled with polyclonal antiserum (Figure 5b). Bacteria carrying this epitope have also been observed inside phagocytic cells (Figure 5c). This indicates that the samll capsule does not impede phagocytosis. When in-vivo grown *B. fragilis* NCTC 9343 populations were labelled with McAb Bf1


(b)
(a)

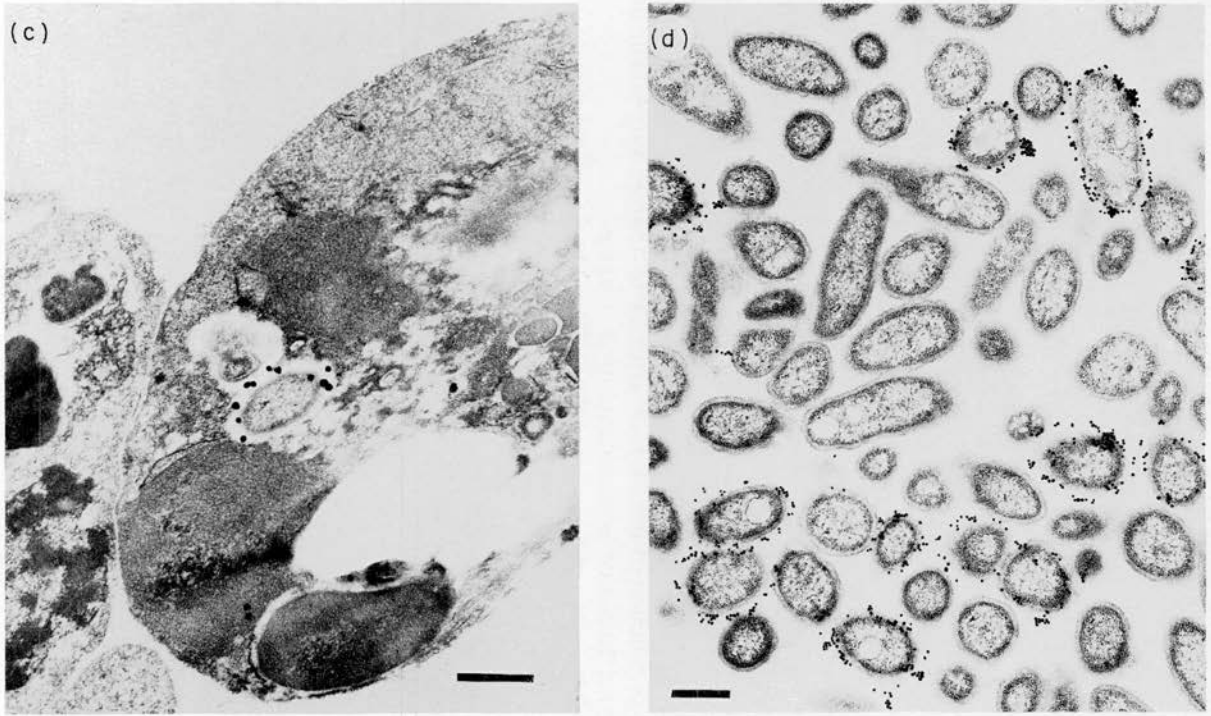


Figure 5. Electron micrographs of in-vivo grown *B. fragilis* NCTC 9343. (a) Labelled with monoclonal antibody Bf4 and anti-mouse IgM gold conjugate. Note heterogeneous labelling of the bacteria. (Reproduced from Reid JH, Patrick S, Tabaqchali S. *J Gen Microbiol* 1987; **133**: 171–179, by permission of the Society for General Microbiology.) (b) Labelled with mouse anti-*B. fragilis* polyclonal antiserum and anti-mouse IgG gold conjugate. Note homogeneous labelling of the bacteria. (c) Labelled with monoclonal antibody Bf4 and anti-mouse IgM gold conjugate. Note labelled bacterium inside phagocytic cell. (d) Labelled with monoclonal antibody Bf1 and anti-mouse IgG gold conjugate. Note heterogeneous labelling of the bacteria.

(Figure 5d), Bf2 and Bf5 (not illustrated) antigenically heterogeneous populations were again observed. The epitopes labelled by these McAb do not appear to be associated with structures identifiable by ordinary staining techniques. Bacteria expressing these epitopes have also been observed inside phagocytic cells.

6. Conclusions

Although the large capsule can impede phagocytosis in in-vitro experiments, it does not seem to play a role in a mouse model of peritoneal infection. This highlights the importance of examining the growth of these bacteria *in vivo*. It also raises the question of whether the mouse or human immune system could already be primed to certain *B. fragilis* surface antigens as a result of its presence in the gut, thus putting bacteria carrying these antigens at an immediate disadvantage in an infection.

B. fragilis populations can vary considerably in the surface structures and antigens produced within one strain. This has important implications in, firstly, studies of phagocytosis, or indeed any aspect of the pathogenesis of this bacterium, in which the possible role of surface structures is being investigated and, secondly, the production of reliable diagnostic tests based on immunological methods. Finally, it is possible that antigenic heterogeneity and the ability to switch from the production of one surface structure to another may be one of the most important virulence determinants of *B. fragilis*.

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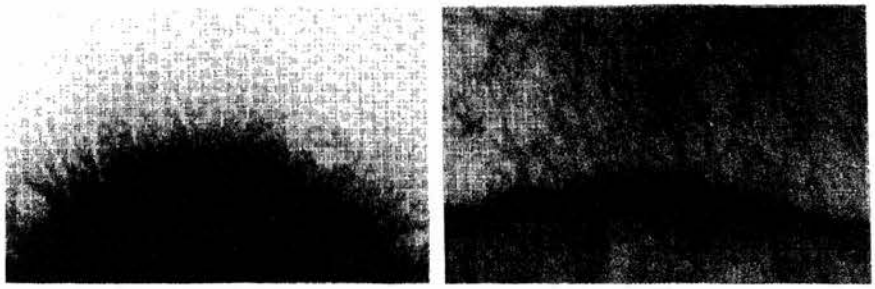

(a) (b)

Figure 1. *B. intermedius*: (a) fibrils on strain 18/23 (×129 000), (b) branched fibrous processes (×95 000).

narrow layer, but in addition produced branched fibrous processes which extended far from the cell wall (Figure 1b).

All strains were hydrophobic: the %HP ranged from 23.8% (±2.7) for strain OMZ227, to 72.6% (±4.2) for strain OMZ327. There was no relationship between %HP and the appearance of the RR staining layer or fibrils. No strain caused significant agglutination of sheep erythrocytes under the conditions described.

Thus, the cell surface of *B. intermedius* is fibrillar, capsulate, hydrophobic and carries a layer of polyanionic surface material. It is not known if the three structures described, which were detected by three different techniques, represent the same surface component.

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2

Iron Limitation and Induction of Congo Red Binding in *Bacteroides fragilis* NCTC 9343

*M. J. Larkin, J. McGuigan and S. Patrick**
*Sub-Department of Microbiology and *Department of Microbiology & Immunobiology, The Queen's University, Belfast, UK*

The ability to chelate iron is an important virulence determinant of pathogenic bacteria because iron is not readily available in body fluids. A number of successful pathogens

produce membrane-bound and excreted chelators in order to trap iron for growth (1). The ability of Gram-negative bacteria to bind congo red dye to their cell surface has been linked to their ability to chelate iron (2,3). Many avirulent strains do not bind congo red; however, their virulence is enhanced by iron supplementation. The possibility of a similar mechanism(s) in *Bacteroides fragilis* has not been investigated although it causes a wide range of infections (4). *B. fragilis* shows an absolute requirement for haem (supplied as haemin) in the medium, with or without iron supplementation (supplied as Fe^{3+}). When grown in defined medium with excess free iron and excess haem, congo red was readily bound. This binding was strongly inhibited by haemin in the assay solution. When grown in haem-limited defined medium, there was an increase in the capacity to bind congo red as the concentration of free iron was lowered. This increase in congo red binding under low-iron conditions was accompanied by the appearance of a 40 000 molecular weight protein in an SDS-PAGE gel of an outer membrane protein preparation. Although iron use by *B. fragilis* appears to be complex, it is postulated that there is an inducible iron uptake mechanism activated under low-iron conditions.

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3

Fusobacteria and Tropical Ulcers

B. Adriaans and B. S. Drasar
London School of Hygiene and Tropical Medicine, London, UK

Tropical ulcer is an acute localized necrosis of the skin and subcutaneous tissues endemic in, but not confined to, the tropics. The initial lesion is a small papule overlying an area of necrotic dermis. The epidermis breaks down to reveal a circumscribed ulcer with regular indurated margins (1). The aetiology of tropical ulcers is obscure. Some authors regard the disease as a non-specific response to skin injury, although there is evidence that the disease is transmissible (2). Reports on the bacteriology of these ulcers have concentrated on aerobic culture in contrast to the plethora of reports mentioning the presence of fusiform bacteria and spirochaetes seen in smears. These are fastidious organisms and thus unlikely to have been detected in culture using the techniques described previously. We attempted to study the role of these anaerobic bacteria in the pathogenesis of tropical ulcers.

We studied 120 patients with tropical ulcer in five locations in the tropics where the condition is prevalent. We isolated several anaerobic organisms from the patients with

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The relationship between cell surface structure expression and haemagglutination in *Bacteroides fragilis*

S. Patrick [a], A. Coffey [a], A.M. Emmerson [a] and M.J. Larkin [b]

Departments of [a] *Microbiology and Immunobiology and* [b] *Biology, The Queen's University of Belfast, Belfast, U.K.*

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Key words: *Bacteroides fragilis*; Fimbria; Capsule; Virulence determinant

1. SUMMARY

Bacteroides fragilis populations were separated according to the size of surface structure. Subculture of the separated populations produced cultures enriched for 3 different structures; a large capsule, a small capsule and an electron-dense layer (EDL). The ability of these subpopulations to haemagglutinate (HA) erythrocytes from a number of species was examined. Populations which produced either a large or a small capsule did not have HA activity, whereas those with an extracellular EDL did. By mixing populations with EDL and those with either the large or small capsule, the degree of HA could be altered. HA was dependent on the proportion of EDL-bearing bacteria present. Fimbriae were not observed on electron microscopy.

2. INTRODUCTION

B. fragilis is the Gram-negative anaerobic bacterium most commonly isolated from clinical infection [1]. The precise virulence determinants of this bacterium have yet to be determined, although surface structures may be involved [2]. Attachment to eukaryotic cells is an important virulence determinant in a number of bacteria [3], and the ability of bacteria to agglutinate erythrocytes has been used as an indication of attachment [4]. Apparently conflicting reports of haemagglutination in *B. fragilis* have suggested that fimbriae [5] and capsule [6,7] are involved. In these studies the bacterial populations were almost certainly heterogeneous with respect to surface structure, as variation in both the size and nature of surface structure occurs within individual populations of *B. fragilis* [8]. 3 apparently different surface structures have been observed by electron microscopy. These are a large capsule, a small capsule and an EDL. Populations enriched for one or other of these structures can be obtained by separation on a Percoll step density gradient and subculture from the gradient interface layers. In the present study the relationship between these different surface structures and agglutination of erythrocytes from a number of species was studied.

Correspondence to: S. Patrick, Dept. of Microbiology and Immunology, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, U.K.

3. MATERIALS AND METHODS

3.1. Bacterial growth conditions

B. fragilis strains were grown to late exponential phase in either defined broth [9] or basal broth [10] at 37 °C in an anaerobic cabinet (Don Whitley) with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Identification was confirmed with the API 20A system. *Escherichia coli* was grown to late exponential phase in nutrient broth, aerobically.

3.2. Separation of bacterial subpopulations

Bacterial populations with different sizes of extracellular structures, as determined by light and electron microscopy, were separated on a four-step Percoll density gradient (20%, 40%, 60% and 80% Percoll) as previously described [11]. Cells from each interface layer (IL) were subcultured into a defined broth. The subcultures from the gradient IL were enriched for cells with the following structures: 0–20% IL, large capsule; 20–40% IL, small capsule; 40–60% IL, small capsule and EDL; 60–80% IL, EDL [8]. The Percoll separation was repeated and the enriched populations then subcultured into either defined broth or basal broth.

3.3. Haemagglutination tests

Washed suspensions of the enriched bacterial populations in phosphate buffered saline were examined for their ability to cause erythrocyte agglutination. Both the rocked tile test and the static settling test as detailed by Old [12] were used with a variety of different erythrocyte species. Bacterial cell numbers were estimated with a Corning colorimeter 252 at A_{600}.

In the rocked tile test 0.04 ml of bacterial suspension (concentration at least 10^{10} cells/ml) was mixed with 0.04 ml of 2% (v/v) erythrocyte suspension on a chilled ceramic tile and agitated by hand. A positive result was recorded if HA occurred within 5 min. In the static settling test, 0.1 ml of bacterial suspension (concentration as specified in the results) was mixed with 0.1 ml of erythrocyte suspension (2% v/v) in the cavities of disposable WHO trays (L.I.P. Equipment and Services Ltd). HA was normally recorded after 1h incubation at 4 °C, the time taken for non-ag-

Table 1

Comparison of haemagglutination of a number of erythrocyte species by *B. fragilis* populations subcultured from the 0–20% [a] and 60–80% [b] interface layers of a step density gradient

Strain	Interface layer	Erythrocyte species								
		Human			Horse	Guinea pig	Rabbit	Sheep	Chicken	Mouse
		O	A	B						
NCTC9343	0–20%	– [c]	–	–	–	–	–	–	–	–
	60–80%	+ [c]	+	+	+	+	+	+	+	+
NCTC9344	0–20%	–	–	–	–	–	–	–	–	–
	60–80%	+	+	+	+	+	+	+	+	+
ATCC23745	0–20%	–	–	–	–	–	–	–	–	–
	60–80%	+	+	+	+	+	+	+	+	+
JC19	0–20%	–			–	–	–	–	–	–
	60–80%	+			+	+	+	+	+	+
GNAB4	0–20%	–	–	–	–	–	–	–	–	–
	60–80%	+	+	+	+	+	+	+	+	+
JC10	0–20%	–			–			–		
	60–80%	–			–			–		

[a] Enriched for large capsule

[b] Enriched for electron dense layer

[c] + Haemagglutination within 5 min; –, no haemagglutination.

glutinated cells to sediment to the centre of the cavity. Sugar inhibition of the haemagglutination was investigated by the addition of an equal volume of 2% (w/v) sugar solution. *E. coli* strain G14 was used as a positive control in the haemagglutination test.

3.4. Electron microscopy

Bacteria were negatively stained with 1% ammonium molybdate and viewed with a Philips 301 transmission microscope. A fimbriate strain of *E. coli*, G14, was used as a positive control.

4. RESULTS

4.1. Rocked tile test for haemagglutination

Of the 6 *B. fragilis* strains tested, none of the subpopulations with the large capsule haemagglutinated, whereas with the exception of strain JC10, subpopulations with the EDL agglutinated all the species of erythrocytes examined (Table 1). None of the following sugar solutions inhibited the haemagglutination of strains NCTC9343 and JC19: D-arabinose, fucose, fructose, galactose, glucosamine, glucose, mannose, ribose or xylose. The addition of Percoll to the erythrocytes did not affect the haemagglutination reaction.

4.2. Static settling test for haemagglutination

The reaction of strains NCTC9343 and ATCC23745 with human A, B and O blood groups was also examined in the static settling test. At bacterial concentrations of 1×10^{10} and 5×10^{9}, only the EDL-enriched subpopulation haemagglutinated (Fig. 1). At cell concentrations of 2×10^{10}, however, subpopulations with the large capsule were apparently positive. This may be due to

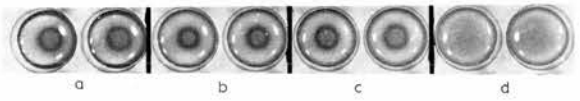

Fig. 1. Static settling test (SST) of HA with human group O erythrocytes and defined broth (DB) grown *B. fragilis* NCTC9343 (bacterial concentration 5×10^{9}/ml). Populations were enriched for (a) large capsule; (b) small capsule; (c) small capsule and EDL; (d) EDL. Note strong positive HA only in (d).

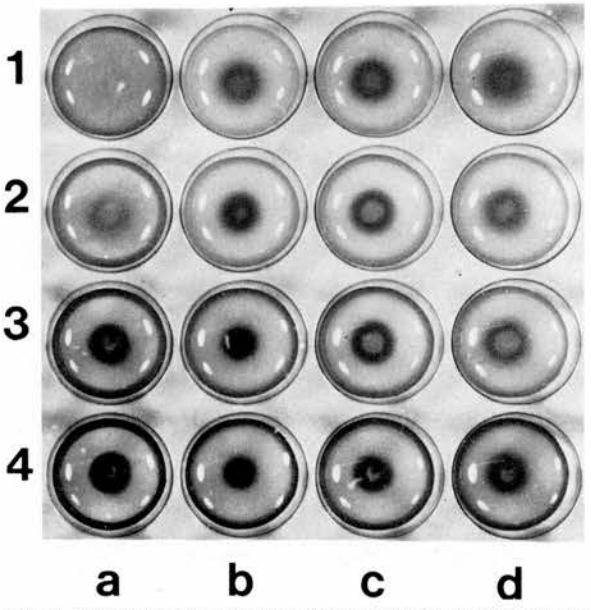

Fig. 2. SST as in Fig. 1 at bacterial concentrations/ml of: row 1, 2×10^{10}; row 2, 1×10^{10}; row 3, 5×10^{9}; row 4, 2.5×10^{9}. Populations were enriched for (a) large capsule; (b) small capsule; (c) small capsule and EDL; (d) EDL. Photographed after positive HA in EDL-enriched population had collapsed inwards (e.g., 1d). Note apparent positive reaction in cavity 1a (large capsule enriched)

the physical size of the large capsule preventing the erythrocytes from settling into the centre of the well. Even after a 2-h incubation period, by which time the layer of erythrocytes agglutinated by the EDL subpopulation had collapsed, the large capsule subpopulation was still apparently positive (Fig. 2). Suspensions of cells with the large capsule, allowed to stand at room temperature for a number of days, did not sediment to the bottom of the container whereas populations without did (not illustrated). Agitation of the sedimented population returned the suspension to its original turbidity. Therefore at bacterial concentrations of 2×10^{10}, and greater, false positive results of haemagglutination may occur.

The four subpopulations were grown in a complex basal medium in which the 0–20% IL (large capsule) enriched bacteria produced smaller capsules [13]. The 0–20% IL enriched population did not haemagglutinate (Fig. 3). There was, however, a gradation in the degree of haemagglutination in the other subpopulations. The EDL population

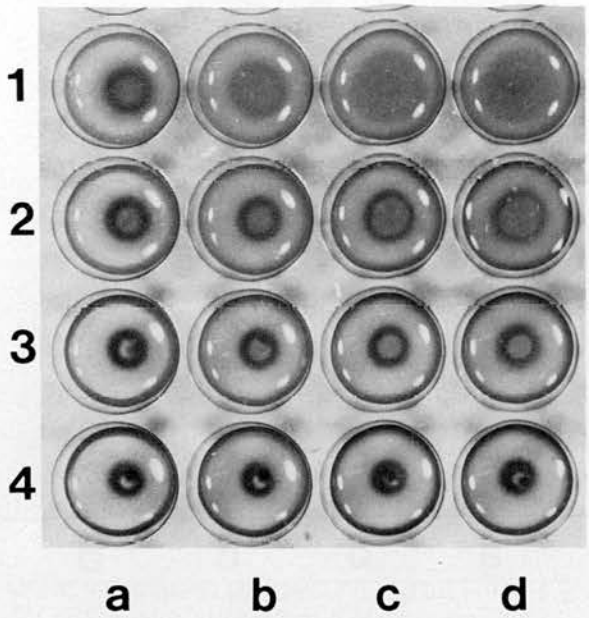

Fig. 3. SST as in Fig. 1 with basal broth grown bacteria at concentrations/ml of: row 1, 1×10^{10}; row 2, 5×10^{9}; row 3, 2.5×10^{9}; row 4, 1.25×10^{9}. Populations were enriched for (a) large capsule; (b) small capsule; (c) small capsule and EDL; (d) EDL. Note the gradation in HA with both the type of enriched population (columns) and doubling dilution of cell concentration (rows)

gave the strongest reaction. This is probably due to a proportion of bacteria from the other IL producing the EDL.

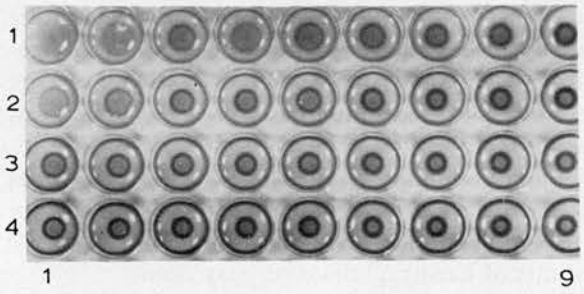

Fig. 4. SST as in Fig. 1 with *B. fragilis* ATCC23745 at bacterial concentrations/ml of: row 1, 1×10^{10}; row 2, 5×10^{9}; row 3, 2.5×10^{9}; row 4, 1.25×10^{9}. The bacterial populations were mixtures of EDL and large capsule enriched bacteria in a series of proportions from 9 parts EDL: 1 part large capsule (line 1) to 1 part EDL: 9 parts large capsule (line 9). Note increased HA (from right to left) with greater proportion of EDL bearing cells.

Subpopulations with the EDL and the large capsule (grown in DM) were mixed in a series of ratios from 1:9 to 9:1 and the ability of the mixtures to haemagglutinate was examined. The degree of haemagglutination correlated with the proportion of bacteria with the EDL (Figs. 4 and 5a). A similar gradation was obtained when the EDL subpopulation was mixed with the small capsule enriched populations (Fig. 5b).

5. DISCUSSION

The results clearly indicate that neither the large capsule or the small capsule are involved in HA, whereas, populations enriched for the EDL do HA. HA tests with populations of *B. fragilis* which are not homogeneous with regard to surface structure may be difficult to interpret as the degree of HA observed in the static settling test correlated with the proportion of EDL-carrying *B. fragilis*.

No fimbriae were observed on electron microscopy, which suggests that the adhesin is closely associated with the cell surface. Possible adhesive factors could include the EDL and outer membrane proteins. An adhesin which can either be carried on fimbriae or associated with the cell surface has been described in *E. coli* [14]. In this instance, the expression of fimbriae and the adhesin was shown to be under separate genetic control. *B. fragilis* fimbriae with no apparent HA activity have been well characterised [15]. If the adhesive factors in *B. fragilis* are subect to phase

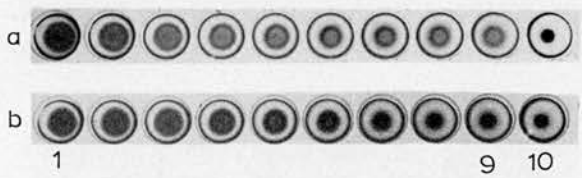

Fig. 5. SST of HA with human group B erythrocytes and DB grown *B. fragilis* NCTC9343 (bacterial concentration 5×10^{9}/ml). The bacterial populations were mixtures of EDL enriched bacteria with (a) large capsule enriched; and (b) small capsule enriched bacteria in a series of proportions from 9 parts EDL (column 1) to 1 part EDL (column 9). Control, column 10 with no bacteria.

and antigenic variation, as in for example *Neisseria gonorrhoea* [16], adhesins of different specificity may be expressed by different strains depending on which selective pressure are acting on the bacterial population present.

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Expression of *Bacteroides fragilis* fimbrial antigen *in vitro* and *in vivo*.
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Expression of *Bacteroides fragilis* fimbrial antigen *in vitro* and *in vivo*

DEBORAH LUTTON,* SHEILA PATRICK,*
JOOP VAN DOORN,† MICHAEL EMMERSON* and
GERRY CLARKE*

**Department of Microbiology and Immunobiology, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, U.K. and †Department of Molecular Microbiology, Faculteit der Biologie, Vrije Universiteit van Amsterdam, Postbus 7161, 1007 MC, Amsterdam, The Netherlands*

Bacteroides fragilis is the Gram-negative anaerobic bacterium most frequently isolated from clinical infections. These include bacteraemia, wound, intra-abdominal and urogenital infections. Populations within individual strains of *B. fragilis* have been shown by transmission electron microscopy (TEM) to be heterogeneous with respect to surface structure. Subpopulations with the following surface structures can be separated by Percoll density gradient centrifugation: (*a*) large capsule; (*b*) small capsule; (*c*) electron-dense layer (EDL); and (*d*) bald cells, i.e. no structure visible outside the outer membrane. Subculture from gradient interfaces results in enrichment of these subpopulations [1]. Fimbriae have been observed on a number of *B. fragilis* strains and are composed of subunits with an apparent molecular mass of 40 000–42 000 Da [2]. In the present study, the relationship between encapsulating surface structures and the presence of fimbrial antigen has been studied both *in vitro* and *in vivo*.

Methods

Microscopy

Light microscopy and thin-section TEM were performed as previously described [1]. Platinum–gold shadowing was carried out in a Balzer BAE 120 high-vacuum coating unit at an angle of 20°.

Immunoblotting

Outer membranes were extracted in 3% (v/v) Sarkosyl, subjected to polyacrylamide-gel electrophoresis (11%, w/v, gel) and blotted on to nitrocellulose. For dot immunoblotting standardized whole-cell suspensions were air dried on to nitrocellulose.

Results and discussion

Microscopy

Light microscopy and thin-section TEM of strain BE3 showed the presence of encapsulating surface structures

Abbreviations used: TEM, transmission electron microscopy; EDL, electron-dense layer.

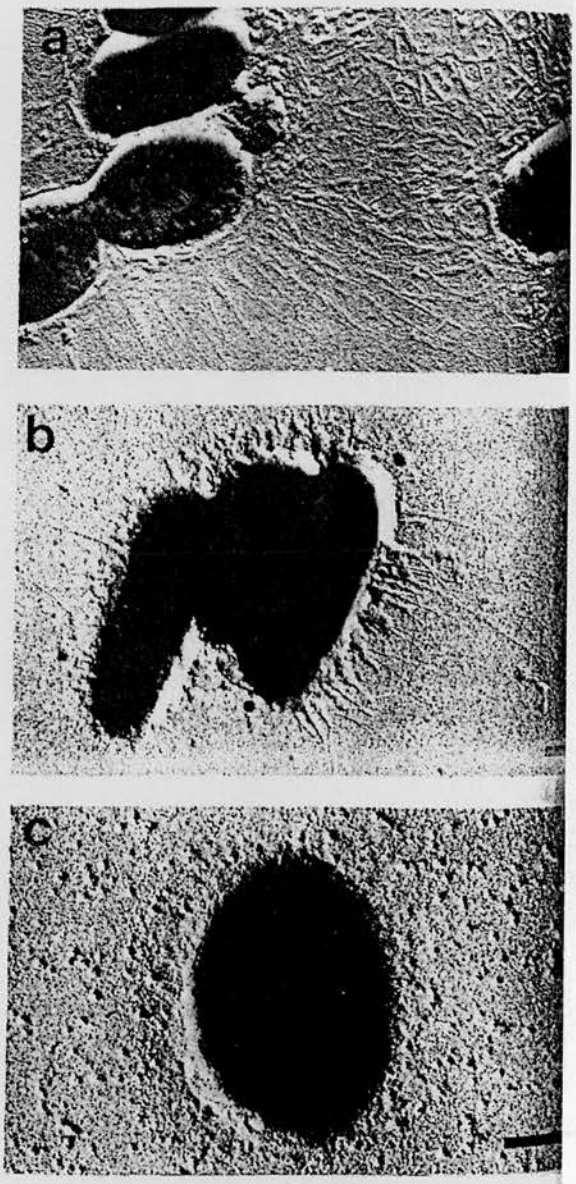

Fig. 1. *TEM of platinum–gold-shadowed Bacteroides strain BE3*

(*a*) Population enriched for large capsules; (*b*) popu enriched for small capsules; (*c*) population enriched f EDL and non-capsulate by light microscopy. Scale b μm.

similar to those already observed on other strains of *B. fragilis* [1]. Platinum–gold-shadowed preparations, when viewed by TEM, showed clumped fibrillar structures (Fig. 1*a*), which corresponded to cells bearing a large capsule by light microscopy and an extensive fibrous network in thin-section TEM. Subpopulations with the small capsule showed shorter clumped fibrillar structures and extended individual strands (Fig. 1*b*).

These structures were not observed on cells which were non-capsulate by light microscopy and have a narrow EDL adjacent to the outer membrane by thin-section TEM (Fig. 1*c*). These structures were not observed in preparations negatively stained with 1% (w/v) ammonium molybdate and observed by TEM.

Immunoblotting

(*a*) *B. fragilis grown in vitro: outer membranes.* Populations of *B. fragilis* strain BE3, enriched for different sizes of encapsulating surface structure as defined by thin-section TEM, were immunoblotted with antiserum to purified fimbriae [2]. Fimbrial antigen was detected predominantly in populations with the small capsule and was either absent or present in reduced amounts in populations enriched for the large capsule, EDL, or bald cells. Immunoblotting of strain NCTC9343, however, showed fimbrial antigen to be present in populations enriched for the large capsule, small capsule and EDL, but absent in populations enriched with bald cells.

(*b*) *B. fragilis grown in vitro: whole-cell suspensions.* Immunoblotting of whole-cell suspensions of enriched populations showed that the fimbrial antigen was associated with the small capsule and the large capsule population of strain BE3 and present in all of the populations, with the exception of bald cells, in strain NCTC9343. Fimbrial antigen was also detected in all the following strains of *B. fragilis*: NCTC9344, 10584; JC6, 10, 11, 14, 15, 17 and 19 (isolated from clinical specimens, Royal Victoria Hospital, Belfast); GNAB92, 93 and 94 (supplied by Dr I. Poxton, Department of Bacteriology, University of Edinburgh); however, no consistent relationship beween the expression of fimbrial antigen and the presence of the small capsule has been found. This may be due to fimbriae of different size altering the mobility of the bacteria during Percoll density gradient separation or contamination by free fimbrial antigen. At present the association of fimbrial antigen with encapsulating surface structure is being pursued using immunogold labelling and TEM.

(*c*) *B. fragilis grown in vivo: outer membranes. B. fragilis* strains NCTC9343, BE3, GNAB92 and JC17 were grown for 3 days in chambers implanted in the mouse peritoneal cavity [3]. Fimbrial antigen was detected in all four strains by immunoblotting. This indicates that it may be involved in the virulence of this bacterium; however, the relative importance of the different surface structures observed to the pathogenic process remains to be determined.

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Outer membrane proteins of *Bacteroides fragilis* grown in vivo

S. Patrick and D.A. Lutton

Department of Microbiology and Immunobiology, The Queen's University of Belfast, Belfast, U.K.

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1. SUMMARY

The outer membrane protein (OMP) profiles of four different strains of *Bacteroides fragilis*, as determined by Coomassie blue stained polyacrylamide gels, were compared after growth in broth culture and in the mouse peritoneal cavity. There was no induction of the expression of large quantities of novel OMP after growth in vivo. Mouse immunoglobulin G and albumin were associated with the bacterial OMP, but could be removed by washing.

2. INTRODUCTION

Bacteroides fragilis is an obligately anaerobic bacterium which is frequently isolated from clinical infection resulting from faecal contamination such as intra-peritoneal infections [1]. Although it is a relatively minor component of the faecal flora it accounts for approximately 40% of the bacterial flora in the irrigation fluid from colonic lavage and colonic tissue specimens. This association with the gut mucosa may contribute to its frequent isolation from gut associated infection [2]. It is likely that the virulence of this bacterium is dependent upon a number of factors including the expression of encapsulating polysaccharides, expression of fimbriae, the ability to adhere to eukaryotic cells, release of extracellular degradative enzymes and the production of enterotoxin [3]. The ability to grow and express virulence determinants in the nutritional conditions present in the host is of prime importance to the successful pathogen. Iron is limiting to bacterial growth in vivo as it is chelated to compounds such as transferrin in body fluids. As a result of this, novel OMP involved in iron uptake mechanisms are induced during growth in vivo and under iron limitation in in vitro laboratory culture in many pathogens [4]. Haem compounds and transferrin can both be used by *Bacteroides* spp. as iron sources in vitro [5].

Therefore to determine if *B. fragilis* produces novel OMP in response to the nutritional conditions in vivo, the expression of OMP was compared after growth in broth culture and in chambers implanted in the peritoneal cavities of mice.

Correspondence to: S. Patrick, Dept. of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvernor Road, Belfast BT12 6BN, U.K.

3. MATERIALS AND METHODS

3.1. Bacterial strains and growth conditions

The following *Bacteroides fragilis* strains were used: strain NCTC9343, the type culture from the National Collection of Type Cultures, U.K.; strain GNAB92, a clinical isolate supplied by Dr I. Poxton, University of Edinburgh, U.K.; strain BE3, a clinical isolate supplied by Dr J. van Doorn, Free University of Amsterdam, NL; and strain JC17, a clinical isolate obtained from the Royal Victoria Hospital, Belfast. Cultures were grown in defined medium broth [6] at 37 °C in an anaerobic cabinet (Don Whitley) with an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Identification was confirmed with the API20A system. Bacteria were also grown in chambers sealed with 0.45 μm membrane filters (Millipore) and implanted into the peritoneal cavity of mice as previously described [7]. Populations enriched for expression of an extracellular electron dense layer visible by electron microscopy, but non-capsulate by light microscopy [8], were used as inocula.

3.2. SDS–PAGE and immunoblotting

Outer membrane protein preparations were obtained by placing bacterial suspensions of approximately $2–4 \times 10^9$ cfu, either unwashed or washed three times by centrifugation and resuspension in 1/4 strength Ringer's solution, in 1 ml of 3% (w/v) *N*-lauroyl sarcosinate (Sarkosyl). The samples were then rotated, end-over-end, for 30 min and centrifuged for 1 h in an Eppendorf centrifuge 5414 at $9980 \times g$ (P.A. Lambert, personal communication, modified from Filip et al. [9]). The supernatant was discarded and the pellets stored at −20 °C until used. Samples were diluted 1 : 1 in SDS-reducing buffer (0.0625 M Tris-HCl, pH 6.8) containing 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue. Samples were heated at 95 °C for 5 min before being applied to the gel.

SDS–PAGE was performed on 9% vertical slab gels using the Laemlli [10] buffer system at a constant current of 50 mA per gel. The gels were then stained for protein with Coomassie blue [11]. For immunoblotting material was transferred onto nitrocellulose membrane after the method of Towbin et al. [12] as detailed in the Bio-Rad Transblot Electrophoretic Transfer Cell instruction manual (Bio-Rad Laboratories, U.S.A.). Gels were equilibrated for 30 min in transfer buffer prior to transfer overnight (18 h) at a constant voltage of 30 V. The membrane was blocked by incubation for 1 h at room temperature in 50 : 50 (v/v) horse serum : phosphate buffered saline (PBS; pH 7.4). The membrane was then washed five times in 0.05% (v/v) Tween-20/PBS and incubated with appropriately diluted rabbit anti-mouse albumin conjugated to horseradish peroxidase (Nordic) or goat anti-mouse immunoglobulin G (H + L) conjugated to horseradish peroxidase (Bio-Rad) in PBS. The membrane was then given five quick washes, followed by 5 × five-min washes in Tween/PBS. Finally, the membrane was placed in freshly prepared diaminobenzidine substrate (0.5 mg/ml in 0.05 M Tris-HCl buffer, pH 7.6) with 3 μl of 30% H_2O_2/ml of buffer added immediately prior to use. The reaction was stopped by washing with distilled water. All the above steps were performed with gentle rocking.

4. RESULTS AND DISCUSSION

The outer membrane protein (OMP) profiles of *Bacteroides fragilis* strains NCTC9343, GNAB92, JC17 and BE3 were examined after three days culture in chambers implanted in the peritoneal cavities of mice and compared with those obtained after growth in defined medium broth. Growth in vivo did not result in the expression of large amounts of novel outer membrane proteins as detected by Coomassie blue staining of polyacrylamide gels (Fig. 1), as bands which appeared only in the in vivo cultured bacteria could be attributed to mouse albumin and mouse immunoglobulin G by immunoblotting (Figs. 2 and 3). Therefore there was no expression of large amounts of high molecular mass proteins in response to the nutritional conditions in vivo. The induction of OMP with a high M_r has been reported to occur with other pathogenic bacteria such as *Escherichia coli* grown in the peritoneal cavity of guinea pigs [13] and *Pseudomonas aeru-*

ginosa taken directly from the lungs of patients with cystic fibrosis [14]. One major 33 kDa band was absent from the in vivo cultured bacteria in Coomassie blue stained gels (Fig. 1). The significance of the loss of the 33 kDa protein is not known. There may, however, be other more subtle differences in OMP expression not detected by this method. A fimbrial subunit of 40–42 kDa, not readily visible on Coomassie blue stained gels, was shown to be produced in reduced amounts under conditions of iron limitation by immunoblotting [15].

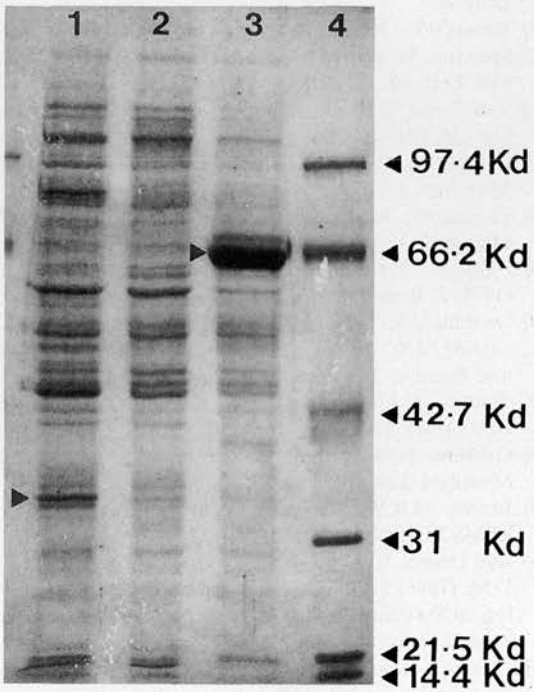

Fig. 1. Coomassie blue stained polyacrylamide gel (PAG) of Sarkosyl extracted outer membranes of *Bacteroides fragilis* (OMP extracts) strain JC17. Lane 1, after growth in defined medium broth; lane 2, after growth in vivo and washed 3× in 1/4 strength Ringer's solution prior to Sarkosyl extraction (washed); lane 3, after growth in vivo and not washed prior to Sarkosyl extraction (unwashed); lane 4, molecular weight standards. Similar results were obtained for strains NCTC9343, BE3 and GNAB92. Note band of M_r 66 kDa is only present in unwashed in vivo grown sample and band of 33 kDa is only present in in vitro grown sample (see arrows). Extract from $2–4 \times 10^9$ bacteria per lane.

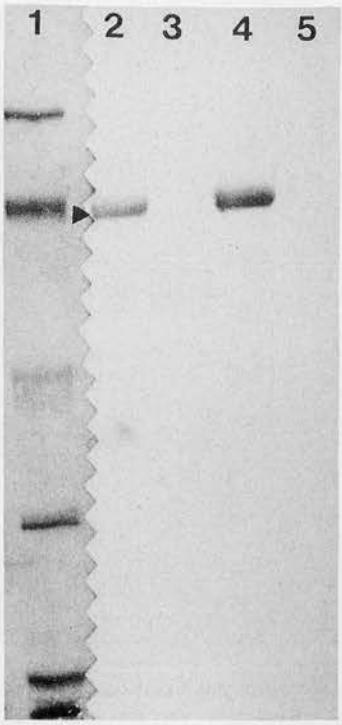

Fig. 2. Immunoblotted PAG of outer membrane extracts reacted with rabbit anti-mouse albumin horseradish peroxidase conjugate. Lane 1, molecular mass standards as in Fig. 1; lanes 3 and 5, bacteria washed; lanes 2 and 4, bacteria unwashed. Strain BE3, lanes 2 and 3; strain NCTC9343, lanes 4 and 5. Note the absence of detectable mouse albumin in the washed samples (see arrows). Similar results were obtained for strains JC17 and GNAB92.

It is interesting that a simple wash by centrifugation and resuspension three times in 1/4 strength Ringer's solution was sufficient to remove the mouse albumin and IgG from the OMP preparation, whereas they were not dissociated from the outer membrane preparation when bacteria were placed directly from the intraperitoneally implanted chamber into 3% Sarkosyl. This indicates that although associated with the outer membrane the albumin and IgG were not specifically bound. This may have implications if immunological methods, such as immunofluorescence or enzyme linked immunosorbent assays, are used to detect bacteria directly in clinical

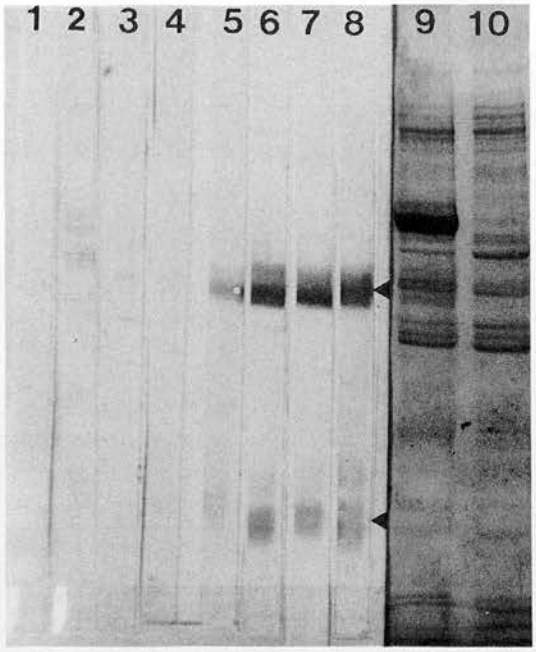

Fig. 3. Immunoblotted and Coomassie blue-stained PAG of OMP extracts. Immunoblots were reacted with goat anti-mouse immunoglobulin G (IgG) heavy and light chain horseradish peroxidase conjugate. Lanes 1–4 and 10, bacteria washed; Lanes 5–8 and 9, bacteria unwashed. Strain BE3, lanes 1, 5, 9 and 10; strain NCTC 9343, lanes 2 and 6; strain JC17, lanes 3 and 7; and strain GNAB92, lanes 4 and 8. Note the absence of detectable heavy and light chain IgG in the washed samples (see arrows).

specimens [16], as host proteins could mask the bacterial antigens.

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Méd Mal Infect. 1990; *20 hors série* : 19-25

BACTEROIDES FRAGILIS SURFACE STRUCTURE EXPRESSION IN RELATION TO VIRULENCE

S. PATRICK* and D. LUTTON*

The possible virulence determinants of *Bacteroides fragilis* include expression of surface structures, release of extracellular enzymes and enterotoxin production (1). Of these, the precise nature of the surface structures expressed has given rise to much confusion. Extracted polysaccharides, generally considered to equate to encapsulating structures, have long been associated with *B. fragilis* virulence as these extracts are known to induce peritoneal abscess formation in animal models (e.g. 2). More recently fimbriae of two different sizes have been described which may be involved in attachment to host cells and surfaces. It is now becoming clear that *B. fragilis* can express a number of different surface structures, identifiable by electron microscopy (EM), which carry antigenically different epitopes.

Structures which encapsulate the bacterium can vary in width from 40 nm to 500 nm as shown by EM of ultra-thin sections. The 40 to 50 nm structures are not seen as classical "capsules" by light microscopy with wet India Ink negative stain (3). Long straight fimbriae of approximately 30 nm (4) and 4-5 nm in diameter (5) have also been observed on some strains by EM negative stain. The 4-5 nm diameter fimbriae have a polypeptide subunit of Mr 40-42 kDa as shown by immunoblotting. There are other reports or fibrillar and fimbrial structures visible by EM (e.g. 6 and 7) which have not yet been fully characterised. There is evidence that the expression of at least some surface structures alter with the growth conditions. It is likely that each individual strain may have the potential to express all or some of these structures in varying proportions.

DEFINITION OF CAPSULES

The size of capsules expressed by individual cells varies within strains of *B. fragilis,* as determined by light microscopy (8). Populations with different sizes of capsules can be separated by Percoll density gradient centrifugation (9). Subculture from the interface layers of the gradient, *in vitro,* will enrich for populations with the different sized capsules (figures 1a, b and c). Bacteria with large capsules remain on top of the Percoll gradient and those with smaller capsules are found lower down. By electron microscopy of ultrathin sections, the large capsule (LC) appears as a large fibrous network of up to 500 nm in width (figures 2a and 2c). The small capsule (SC) appears as a marginal fibrous network of up to 50 nm in width by thin section EM (figures 2a and 2d). Populations which seem to be non-capsulate by light microscopy have a marginal electron dense layer (EDL) of up to 40 nm by thin section EM (figures 2a and 2d). The appearance of the LC and SC is greatly enhanced by ruthenium red stain ; however, the EDL is clearly visible without ruthenium red (9). After platinum/gold shadowing of whole bacteria the LC and SC have a fibrillar appearance by EM (figures 3a and b), whereas the EDL has no associated fibrillar structures (figure 3c). Populations enriched for the LC, SC and EDL will gradually revert to mixed populations with successive daily subculture.

It is therefore possible to define three apparently distinct capsules within individual strains of *B. fragilis :* a large capsule, a small capsule and an electron dense layer. These may have a very different appearance depending on which microscopical techniques are used to observe them and, in the case of the EDL, may be only visible by electron microscopy.

EVIDENCE FOR ANTIGENIC HETEROGENEITY

We have prepared a number of different monoclonal antibodies (MAbs) specific for *B. fragilis* surface polysaccharides. These have shown antigenic differences between the populations enriched for the dif-

* Laboratoire de Bactériologie, Centre Hospitalier, 1, av. de Trésum, F-74011 Annecy.

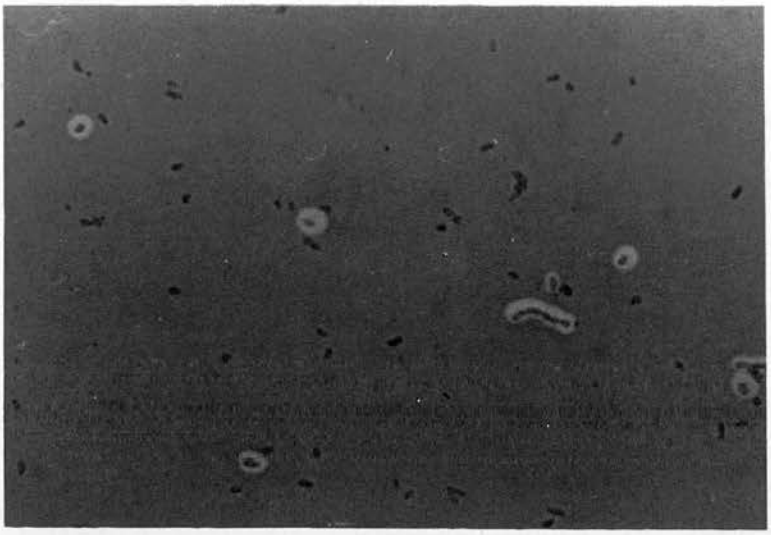

Figure 1a

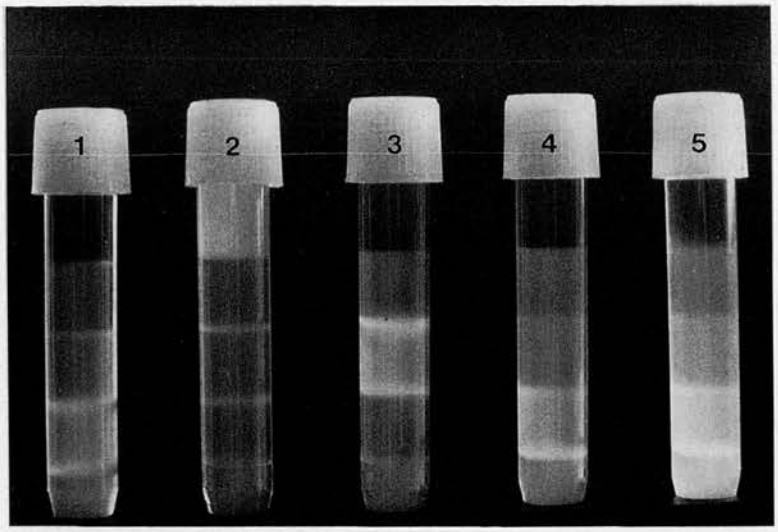

Figure 1b

Figure 1 :
a) Light micrograph of *Bacteroides fragilis* laboratory stock culture, stained with eosin-carbol fuchsin. Note the presence of bacteria with large capsules, small capsules and apparently non-capsulate bacteria.
b) Four step (20, 40, 60 and 80%) Percoll density gradients after centrifugation in a bench centrifuge. Tube 1, pattern of bands when laboratory stock culture was centrifuged. Tubes 2-5, separation, on the same gradient, from first subculture into broth from each interface : 2, 0-20% (top) interface; 3, 20-40% interface; 4, 40-60% interface; 5, 60-80% interface. Note enrichment for populations which band at the different interfaces after one overnight subculture. (Reproduced from Patrick S., Reid J.H. and Coffey A.; *J. Gen. Microbiol.* 1986, *132*, 1099-1109, by permission of the Society for General Microbiology).
c) Light micrographs of enriched populations stained with eosin-carbol fuchsin taken from: 1, 0-20% interface; 2, 20-40% interface; 3, 40-60% interface; 4, 60-80% interface. Note the enrichment for large capsule, small capsule and apparently non-capsulate bacteria.

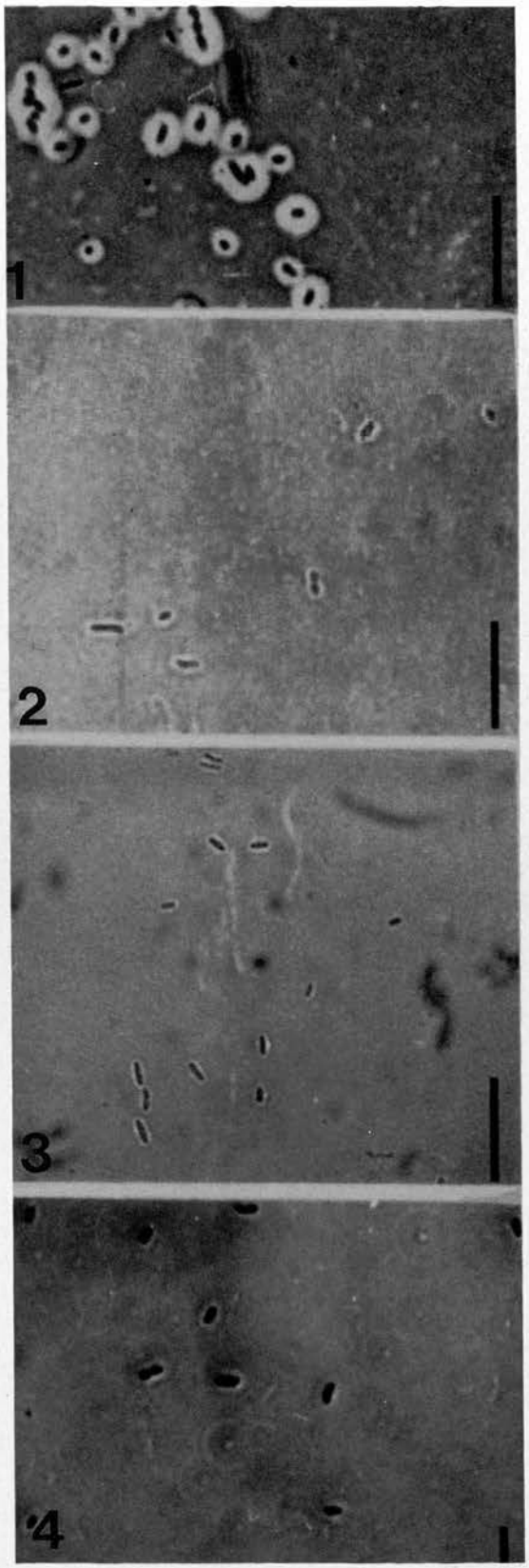

Figure 1c

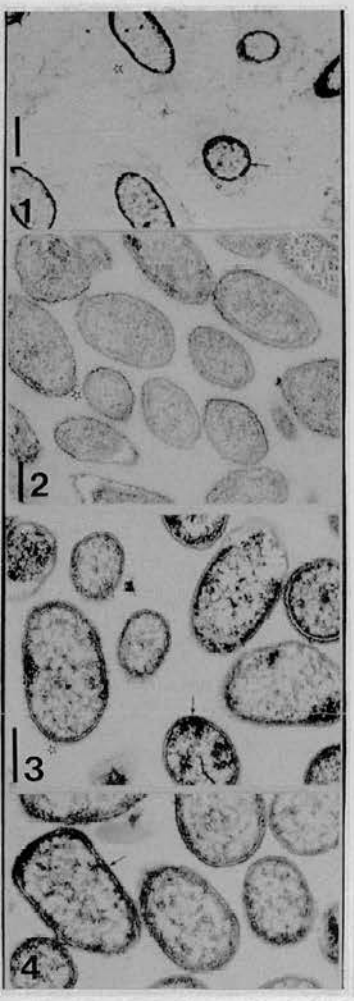

Figure 2a

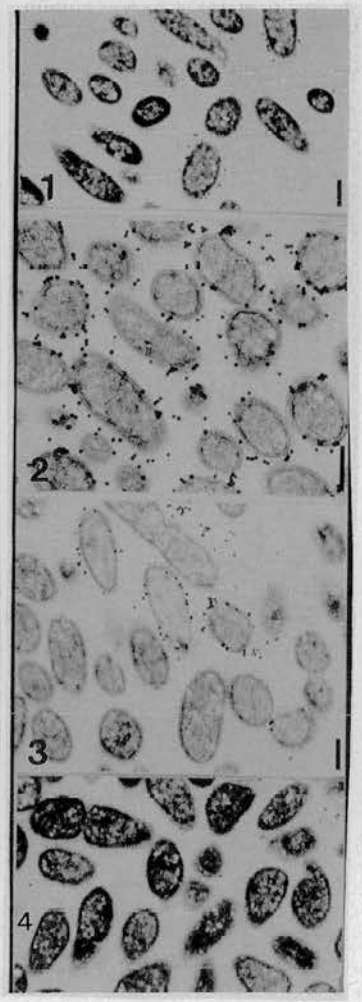

Figure 2b

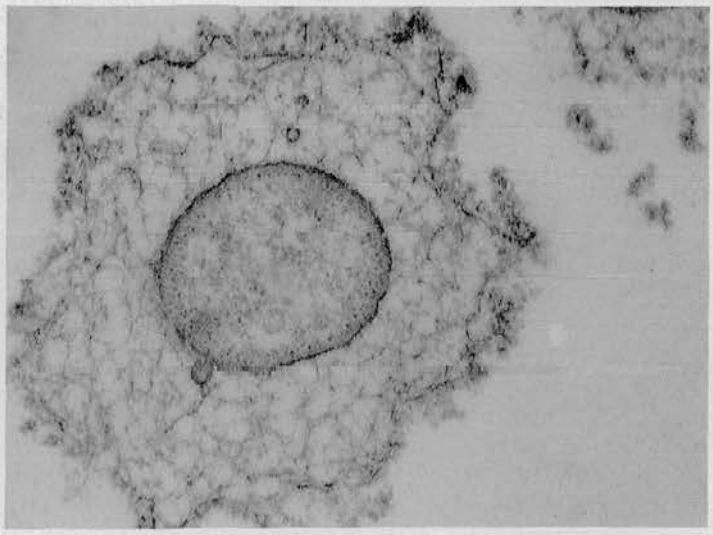

Figure 2c

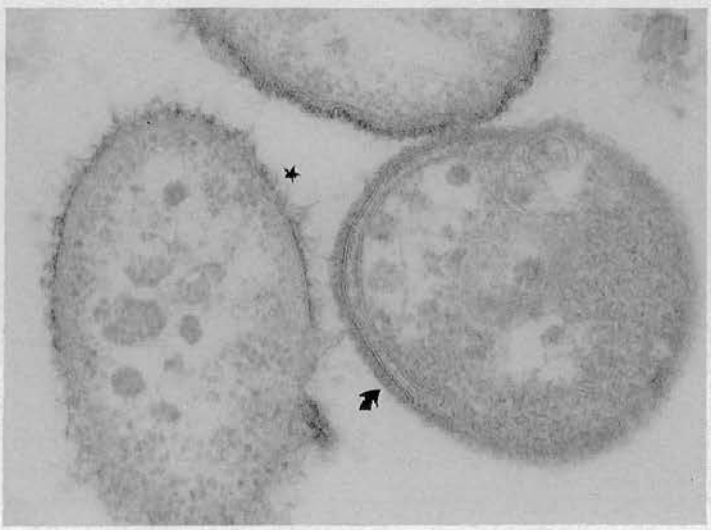

Figure 2d

Figure 2 :
a) Electron micrographs (ultra-thin section) of enriched *B. fragilis* populations taken from: 1, 0-20% interface, note the large fibrous network or capsule; 2, 20-40% interface, note the marginal fibrous network or small capsule; 3, 40-60% interface, note some cells with marginal fibrous network (*) and some with a marginal electron dense layer (arrow); 4, 60-80% interface, note bacteria which appeared to be non-capsulate by light microscopy have a marginal electron dense layer. (Reproduced from Reid J.H., Patrick S. and Tabaqchali S.; *J. Gen. Microbiol.* 1987, *133*, 171-179, by permission of the Society for General Microbiology).
b) Equivalent populations to those illustrated in Figure 2a prepared for electron microscopy and immunogold labelling which, although retaining the antigenicity, does not allow visualisation of the fibrous network or electron dense layer. All populations labelled with murine monoclonal antibody BF4. Note association of labelling with the small capsule population (2) and lack of labelling in the electron dense layer population (4). (Reproduced from Reid J.H., Patrick S. and Tabaqchali S.; *J. Gen. Microbiol.* 1987. *133*, 171-179, by permission of the Society for General Microbiology).
c) Electron micrograph of populations enriched from the 0-20% interface layer at higher magnification. Note the large fibrous network or capsule.
d) Electron micrograph of populations enriched from the 40-60% interface layer. Note the distinct appearance of the marginal fibrous network or small capsule (*) and the marginal electron dense layer (arrow).

Figure 3 : Electron micrographs of platinum-gold shadowed preparations of whole *B. fragilis* cells:
a) 0-20% interface layer enriched population. Note fibrillar appearance of the large capsule.
b) 20-40% interface layer enriched population. Note short and extended fibrills associated with the small capsule.
c) 60-80% interface layer enriched population. Note the absence of fibrillar structures. (Reproduced from Lutton *et al*, Biochem. Soc. Trans. 1989, 17, 758-759, by permission of the Biochemical Society).

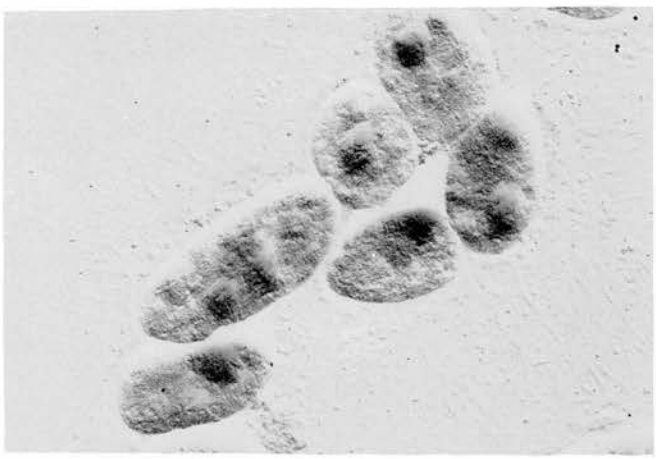

Figure 3a

Figure 3b

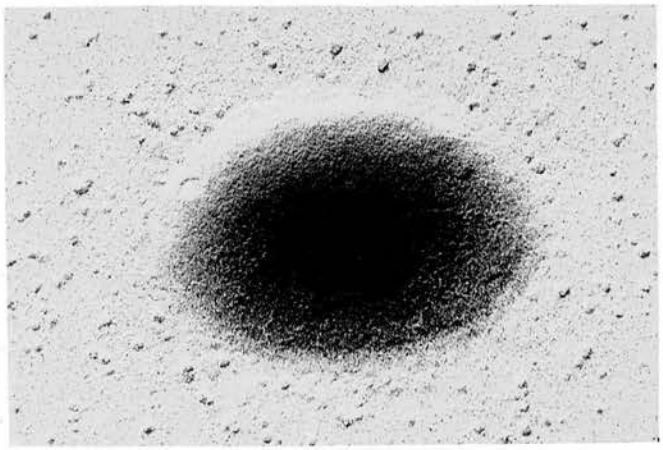

Figure 3c

ferent capsular types. The different capsules observed by EM are therefore not simply the result of differences in the quantity of capsular material present. One of these MAbs specifically labels the small capsule (10). This indicates that the small capsule is antigenically different from the EDL, although similar in size (figure 2b). The same result by can be illustrated by dual colour fluorescent labelling and examination with a fluorescence microscope. Populations labelled with both murine MAb BF4 and a rabbit polyclonal antiserum show quite clearly that BF4 does not label the EDL population (figure 4a). Anti-polysaccharide MAbs, for example BF5, which do not label the small capsule population, but do label a proportion of cells within the EDL (figures 4b and 4c) and LC populations (not illustrated) have provided further evidence for the antigenic heterogeneity of *B. fragilis*.

It is possible to quantify the proportion of bacteria labelled within the populations by counting fluorescently labelled bacteria by flow cytometry in a Coulter electronically programmable individual cell sorter (EPICS V) as illustrated in figure 5. This confirms the microscopical observations, again showing that the SC and EDL are antigenically different.

How the polysaccharides, recognised by these MAbs, relate to the rough lipopolysaccharide (LPS), smooth LPS, common polysaccharide antigen and high molecular weight polysaccharide which can be detected immunochemically in purified polysaccharide preparations (11) is yet to be determined. These labelling results, however, do provide strong evidence for structural heterogeneity within individual strains of *B. fragilis* to the extent that the SC and the EDL are distinct. The results also illustrate a degree of antigenic heterogeneity only detectable by immunological labelling.

FIMBRIAL EXPRESSION VERSUS CAPSULE EXPRESSION

The expression of the 40-42 kDa polypeptide subunit of the 5nm diameter fimbriae (figure 6) was examined in populations enriched for the various encapsulating structures. Results indicate that fimbrial antigen is not expressed in *B. fragilis* strain BE3 when the LC is expressed, although in other strains the relationship between expression of encapsulating structures and the fimbriae was not as distinct (12).

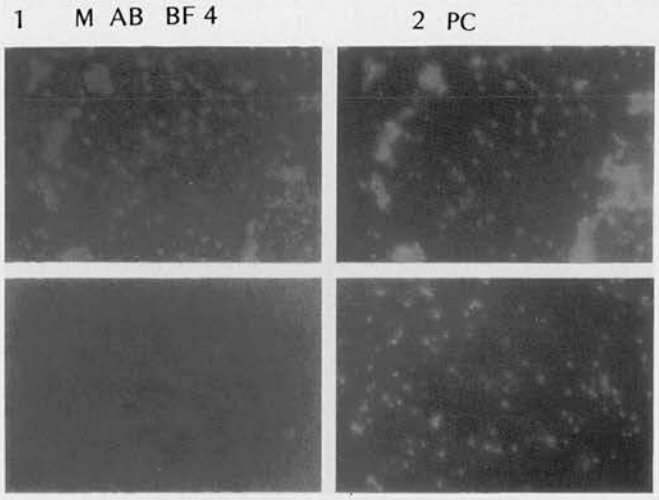

Figure 4a

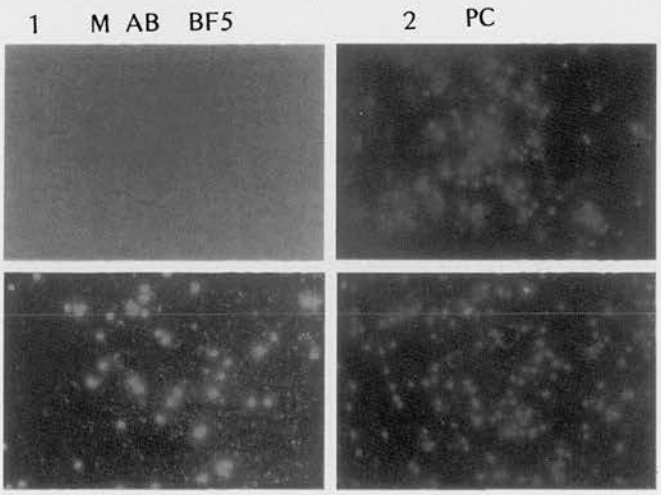

Figure 4b

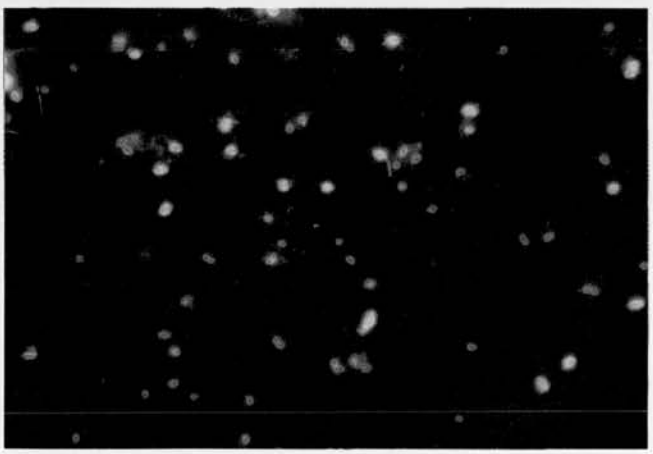

Figure 4c

Figure 4 : Dual colour fluorescence labelling of small capsule enriched (SC) and electron dense layer enriched (EDL) populations.
a) 1. - Murine monoclonal antibody BF4 (M AB BF4) and fluorescein conjugated secondary antibody;
2. - Rabbit anti-*B. fragilis* polyclonal antiserum (PC) and a rhodamine conjugated secondary antibody. Note green fluorescence of monoclonal antibody labelling only the small capsule enriched population.
b) 1. - Monoclonal antibody BF5 (M AB BF5) and rhodamine conjugated secondary antibody;
2. - Rabbit anti-*B. fragilis* polyclonal antiserum and fluorescein conjugated secondary antibody. Note lack of labelling of the small capsule population and red fluorescence of monoclonal antibody which labels only a proportion of the electron dense layer enriched population.
c) Electron dense layer enriched population labelled with monoclonal antibody and polyclonal antiserum. Photographic double exposure of the same feild of view to show both red and green fluorescence together. Note that only a proportion of the total population are labelled with the monoclonal antibody (red).

Recent data indicate that a population of strain BE3, initially enriched for expression of the LC, gradually begins to express fimbrial antigen as the population reverts to a culture with mixed encapsulating structures (unpublished result). Therefore in this strain, expression of the large capsule was not associated with the detection of fimbrial antigen by immunoblotting. This is further evidence for the heterogeneous nature of surface structure expression in *B. fragilis*.

RELATIONSHIP WITH VIRULENCE

It is not yet known in what precise way these different surface structures relate to the virulence and pathogenesis of *B. fragilis*. Expression of the 30 nm fimbriae (4) is associated with haemagglutination and may play a role in the specific attachment of *B. fragilis* to host cells. The narrower fimbriae of approximately 4-5 nm are not associated with haemagglutination (5), although the fimbrial polypeptide subunit of 40-42 kDa has been detected by immunoblotting in bacteria which were grown intra-peritoneally in mice (12). Although the LC impedes phagocytosis *in vitro* (13), paradoxically it is selected against during growth in the mouse peritoneal cavity in the presence of phagocytes (3). *B. fragilis* with the LC survive better under aerobic conditions and are not involved in haemagglutination reactions (14, 15).

The possible relationship of the SC to virulence has not yet been fully investigated. Immunogold labelling with the MAb specific for the SC has shown that although a proportion of bacteria growing *in vivo* express the small capsule, there is no strong selection in favour of this population.

The EDL population is associated with the ability to agglutinate erythrocytes from a number of species (15) and is more hydrophobic than the LC population (unpuplished result) which suggests that it may be involved in attachment mechanisms. The EDL population is also resistant to the killing action of nor-

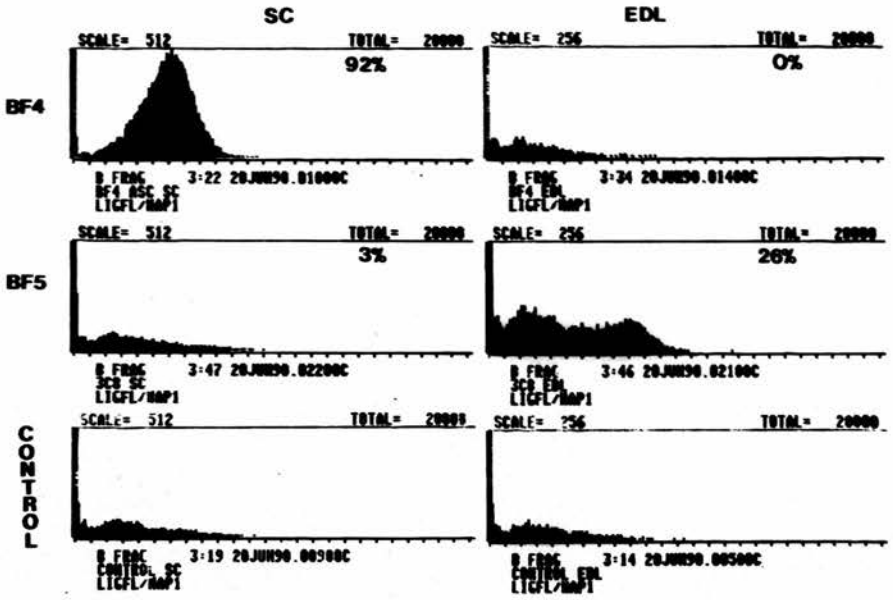

Figure 5

Figure 6

Figure 5 : Flow cytometric analyses of small capsule (SC) and electron dense layer (EDL) enriched populations labelled with monoclonal antibodies BF4 and BF5. Control: populations labelled with only the fluorescein conjugated secondary antibody. Note that graphs are only significantly higher than control for BF4 with the SC population and BF5 with the EDL population. Total number of bacterial cells fluorescing within the population is expressed as a percentage.

Figure 6 : Electron micrograph of negatively stained whole cell of *B. fragilis* to illustrate the 4-5 nm diameter fimbriae.

mal human serum, as determined in an *in vitro* assay (13), although it does not confer resistance to phagocytosis.

CONCLUSION

It therefore seems that *B. fragilis* surface structures and antigens are extremely varied. Expression of different structures may depend on the selective pressures acting on a given population at a given time during an infection. This makes it difficult to interpret results of studies of the relationship between surface structures and virulence mechanisms where the structures present within the population have not been clearly defined. Not only are there antigenic differences between strains but a high degree of heterogeneity of surface antigen expression occurs within individual strains. It is likely that much of the apparent confusion as to the nature of the surface structures expressed by *B. fragilis* and their possible relationship with virulence stems from their inherent heterogeneity.

ACKNOWLEDGEMENTS

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RÉSUMÉ

BACTEROIDES FRAGILIS. EXPRESSION DES STRUCTURES DE SURFACE EN RELATION AVEC LA VIRULENCE

Les déterminants possibles de la virulence de *B. fragilis* incluent l'expression des structures de surface, la libération d'enzymes extracellulaires et la production d'entérotoxine. A partir de ceci, la nature précise des structures de surface a conduit à beaucoup de confusion. Il devient maintenant évident que *B. fragilis* peut exprimer un certain nombre de structures de surface, identifiables en microscopie électronique, qui portent des épitopes antigéniquement différents. Les structures encapsulant la bactérie peuvent avoir une grosseur variant de 40 à 500 nm, comme le montre la section ultra-mince en microscopie électronique. En microscopie optique par coloration à l'encre de Chine les structures de 40 à 50 nm ne sont pas vues comme des capsules classiques. De longs fimbriae rectilignes d'approximativement 30 nm et 5 nm de diamètre ont aussi été observés chez certaines souches par coloration négative en microscopie électronique. Les fimbriae de 5 nm de diamètre ont une sous-unité polypeptidique de Mr 40-42 Kd, comme on peut le voir en immunoblotting. D'autres structures fibrillaires ou fimbriales ont été rapportées. Cela prouve que l'expression d'au moins certaines de ces structures se modifie en fonction des conditions de croissance. Chaque souche individuelle peut avoir le potentiel pour exprimer toutes ou certaines de ces structures dans des proportions variées.

Mots clés : *B. fragilis* - Facteurs de virulence - Capsule.

SUMMARY Bacteroides fragilis *can express a number of different surface structures identifiable by electron microscopy. These include (a) two different types of fimbriae, 4-5 nm and 30 nm in diameter respectively, and (b) three apparently different encapsulating structures, a large capsule, small capsule and electron dense layer. Populations expressing different types of capsule can be enriched for by Percoll density gradient centrifugation. A monoclonal antibody, BF4, labels an epitope on the surface of the small capsule population, but not either the large capsule or electron dense layer populations. A number of monoclonal antibodies have been characterised which label a proportion of bacteria within the large capsule and electron dense layer populations but not the small capsule population. It therefore seems that the small capsule and electron dense layer are distinct. Monoclonal antibody BF5 only labelled a proportion of the bacteria within populations enriched for either the large capsule or electron dense layer. This was quantified by counting the fluorescently labelled bacteria by flow cytometry in an electronically programmable individual cell sorter (Coulter EPICS V). In* B. fragilis *strain BE3, expression of the large capsule was not associated with detection of fimbrial antigen by immunoblotting. Therefore there is evidence for a high degree of structural and antigenic variability within individual strains of* B. fragilis. *This may be relevant to the virulence of this bacterium.*

Keys-words : B. fragilis - *Virulence factors - Capsule.*

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MEDECINE
ET MALADIES
INFECTIEUSES

ERRATUM

Dans le numéro spécial de Décembre 1990 sur :

LES ANAÉROBIES
Actualités en 1990
I.C.I.D. Montréal – Juillet 1990

Une erreur s'est glissée dans l'adresse des auteurs de l'article :

« *BACTEROIDES FRAGILIS* SURFACE STRUCTURE EXPRESSION IN RELATION TO VIRULENCE »

S. PATRICK* and D. LUTTON*

En bas de la page 19, colonne de gauche, il faut lire :

* Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK.

Brook, I, Gerard A, Lambe DW, Maclaren DM, Patrick S and Sebald M.
1990.
Pathogenie des infections a bacteries anaerobies.
Medecine et Maladies Infectieuses 20 hors serie, 45-47.

Méd Mal Infect. 1990; *20 hors série* : 45-47

TABLE RONDE

PATHOGENIE DES INFECTIONS A BACTERIES ANAEROBIES

Participants : I. BROOK, A. GERARD, D.W. LAMBE, D.M. MACLAREN, S. PATRICK, M. SEBALD.

● LES INFECTIONS A GERMES ANAEROBIES SONT RAREMENT MONOMICROBIENNES. UNE BACTERIE PEUT-ELLE, SEULE, DETERMINER UNE INFECTION ?

◆ Les bactéries anaérobies sont reconnues comme fréquemment responsables d'infections. Elles sont le plus souvent isolées en association avec des bactéries aérobies : ces infections sont considérées comme mixtes. Elles se développent au sein de différents tissus : tête et cou, tractus respiratoire, tractus gastro-intestinal, tractus génital féminin, peau et tissus mous. Il y a quelques années on considérait que les infections dans lesquelles étaient impliquées des bactéries anaérobies étaient obligatoirement mixtes. Actuellement la preuve est faite qu'une bactérie anaérobie est capable de déclencher à elle seule une infection. Il peut s'agir d'une infection mixte au départ, qu'une antibiothérapie sélective anti-aérobie a transformée en une infection n'impliquant que des bactéries anaérobies. L'étude des *Peptostreptococcus* montre qu'ils peuvent, seuls, déterminer des infections allant de la surinfection d'ulcères de jambe jusqu'à la septicémie. *Bacteroides fragilis,* et très exceptionnellement *B. melaninogenicus,* sont capables aussi de déclencher seuls des infections septicémiques. Pour traiter les infections mixtes, une antibiothérapie qui épargne les bactéries anaérobies conduit à un échec souvent désastreux. On peut citer de nombreux exemples : ostéomyélites à *Staphylococcus epidermidis* et *B. fragilis,* infections abdominales avec abcédation secondaires, etc... Des infections expérimentales peuvent être déclenchées chez l'animal par l'injection de bactéries anaérobies seules, provenant de cultures. En utilisant *B. fragilis,* on constate que les souches encapsulées n'ont pas besoin de l'aide des germes aérobies pour exprimer leur pathogénicité. Il est établi que les bactéries anaérobies peuvent être seules responsables d'infections. Leur rôle est très sous-estimé mais les conséquences cliniques d'une antibiothérapie inadaptée sont toujours graves.

● LES ESPECES BACTERIENNES ANAEROBIES SONT LARGEMENT MAJORITAIRES AU SEIN DE LA FLORE DIGESTIVE. COMMENT EXPLIQUER L'ABSENCE DE CORRELATION ENTRE L'IMPORTANCE QUANTITATIVE DE CES ESPECES ET LA PREPONDERANCE QUALITATIVE DE CERTAINES ESPECES EN PATHOLOGIE INFECTIEUSE ?

◆ Effectivement, les bactéries anaérobies majoritaires dans les cavités naturelles ne sont pas les plus fréquemment rencontrées en pathologie infectieuse. Certaines propriétés sont nécessaires à l'expression de la virulence :

Propriété d'adhésion

Les capacités de fixation de *B. fragilis* à la muqueuse intestinale lui confèrent un avantage majeur et permettent la première étape de l'infection, l'invasion. Chez 10 patients ayant subi des lavages intestinaux pré-opératoires, l'étude du liquide de lavage ne met en évidence que de rares *Bacteroides* alors que 50% des bactéries restant fixées sur la muqueuse intestinale sont des *B. fragilis.* Cette fixation rend compte d'une des difficultés techniques rencontrées lors de l'analyse quantitative des flores intestinales : les bactéries prédominantes ne représentent en réalité que les bactéries les plus facilement isolées ! Les propriétés d'adhésion d'une espèce conduisent à un biais dans son estimation quantitative.

La capsule

La présence d'une capsule polysaccharidique, constante chez *B. fragilis,* est un facteur de virulence essentiel. La structure de cette capsule fait l'objet de nombreux travaux, notamment en microscopie électronique. La meilleure technique d'examen demeure

la microscopie à contraste d'interférence différentiel permettant d'observer *B. fragilis* en milieu liquide.

C'est une structure variable. Certaines formes seulement conférant la virulence : c'est aux capsules de grandes tailles que sont attachés les caractères de pathogénicité. La capsule inhibe la migration des macrophages et supprime la phagocytose : la survie des *Bacteroides* est alors prolongée. Certains tests effectués pour étudier le rôle de la capsule donnent des résultats contradictoires. Si des *Bacteroides* sont mis en culture 18 heures en présence de clindamycine, le pourcentage de bactéries phagocytées passe de 30% à 100%. En utilisant la ciprofloxacine, dénuée de toute activité vis-à-vis des *Bacteroides*, on constate une diminution de la phagocytose de 50%. Ceci pourrait expliquer des divergences entre les résultats des tests *in vitro* et l'efficacité thérapeutique *in vivo*. L'effet d'un antibiotique sur la phagocytose doit être considéré avec autant d'attention que son effet bactéricide.

Adaptabilité

Les constituants de l'enveloppe et de la surface bactérienne se modifient à l'origine d'un processus d'adaptation, aux mécanismes de défenses immunitaires entre autres. Le chimiotactisme et la phagocytose peuvent être inhibés par d'autres facteurs que la capsule (acide succinique).

Les toxines

La production d'exotoxine est reconnue depuis longtemps parmi les bactéries anaérobies *(C. botulinum, C. tetani, C. difficile)*. Les endotoxines liposaccharidiques existent chez les bactéries anaérobies comme chez les aérobies mais elles apparaissent moins actives (les chocs septiques sont moins fréquents). Certaines espèces produisent également d'autres enzymes : collagénase de *B. melaninogenicus*, entérotoxine de *B. fragilis* qui serait responsable de diarrhée chez l'homme, superoxyde dismutase qui lui confère une certaine résistance à l'oxygène.

● EST-CE QUE LE ROLE DES BACTERIES ANAEROBIES DANS LES INFECTIONS EST SOUS-ESTIME ? QUELLES EN SONT LES RAISONS ?

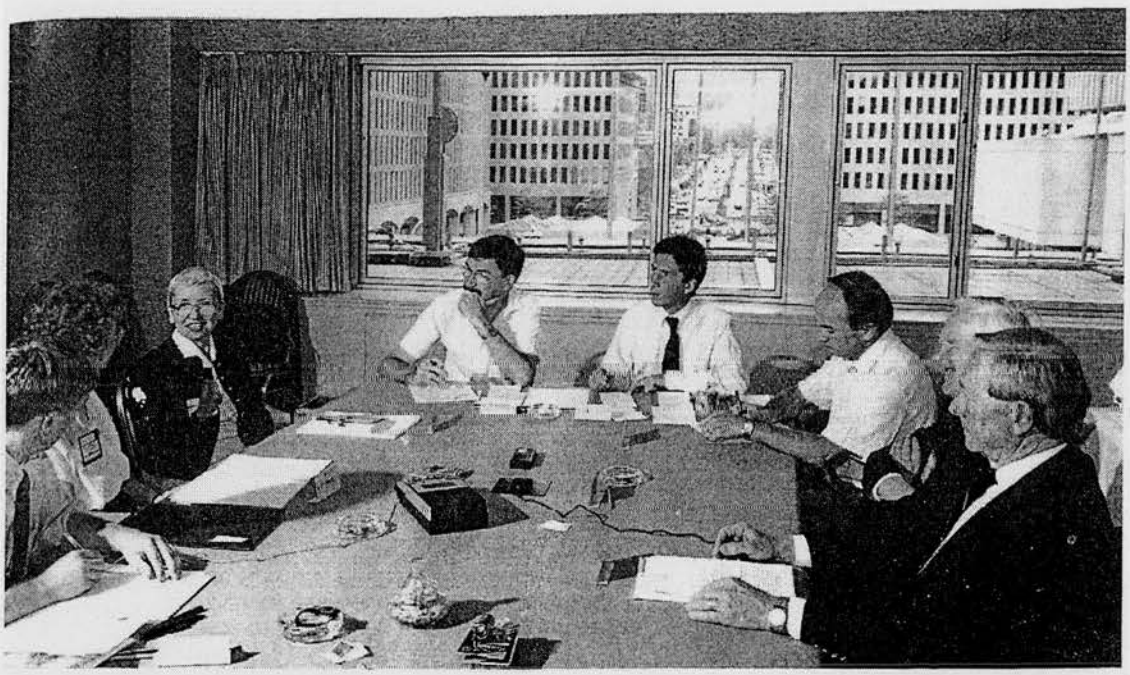
◆ Les difficultés techniques que rencontre le laboratoire pour isoler les bactéries sont nombreuses. La toxicité de l'oxygène pour certaines espèces est telle que leur isolement est impossible si certaines conditions de prélèvement (la fixation des bactéries sur le site infectieux peut être étroite) et d'acheminement ne sont pas remplies. Leur croissance sur les milieux de culture est très lente comparée à celle des bactéries aéro-anaérobies facultatives qui peuvent inhiber la croissance des bactéries anaérobies stricts en acidifiant les milieux. Les techniques de culture doivent être parfaitement adaptées à leur métabolisme (chambre anaérobie). Toute hémoculture doit comporter un flacon en anaérobiose; il est également souhaitable d'utiliser un flacon d'hémoculture contenant un agent hypertonique, ce qui permet d'isoler les bactéries à paroi déficiente. On estime à 10% la fréquence des hémocultures positives à bactéries anaérobies. Dans les flores polymicrobiennes, il y a des phénomènes d'antagonisme qui restent encore mal connus.

● EN PRATIQUE, NE FAUDRAIT-IL PAS PRESCRIRE UNE ANTIBIOTHERAPIE ACTIVE SUR LES GERMES ANAEROBIES CHAQUE FOIS QU'ON SUSPECTE LEUR RESPONSABILITE DANS UNE INFECTION, SANS ATTENDRE UNE REPONSE HYPOTHETIQUE, ET OBLIGATOIREMENT TARDIVE, DU LABORATOIRE ?

◆ Malgré toutes ces difficultés, le maximum doit être fait dans les laboratoires de microbiologie clinique pour isoler les bactéries anaérobies. Mais il est vrai que ne pas prescrire un anti-anaérobie parce que le laboratoire n'a pas isolé la bactérie, peut avoir des conséquences graves. Pour y voir plus clair, envisageons certaines situations cliniques :

Infections du pied chez le diabétique

La présence des bactéries anaérobies dans ces infections est extrêmement fréquente : 90% des cas (le réservoir de ces germes est cutané; l'hygiène des pieds du diabétique doit être rigoureuse). L'évolution dramatique vers l'amputation pourrait souvent être évitée. L'antibiothérapie ne vise que l'éradication des bactéries aérobies, les anaérobies provoquant une lente mais inexorable destruction de l'os. Les bactéries responsables peuvent être mises en évidence si le prélèvement est soigneux mais il est difficile : l'infection se développe sous la forme d'une multitude de micro-abcès qui peuvent chacun contenir des flores différentes. Il faut gratter longuement l'os avant la mise en culture, pour détacher les germes.



Au total, 45 espèces différentes ont été isolées : les cocci anaérobies sont les plus fréquents, mais 8 à 10 jours sont nécessaires à leur isolement, on trouve aussi *B. fragilis* et d'autres *Bacteroides*. Dans ce contexte il apparaît légitime de prescrire une antibiothérapie dont le spectre couvre à la fois les bactéries aérobies et anaérobies.

Infections génitales féminines

Les infections pelviennes sont mixtes le plus souvent. Différentes espèces de *Bacteroides* sont impliquées *(B. fragilis, B. bivius, B. disiens)*. Les capacités d'adhésion des bactéries anaérobies à la muqueuse jouent un rôle très important.

Infections sur prothèse

De nombreuses infections sur prothèse de hanche sont provoquées par des *Peptostreptococcus*, dont la croissance, aussi bien sur les milieux de cultures que dans les tissus infectés, est très lente. Les patients restent asymptomatiques pendant longtemps, l'infection s'installant à bas bruit. L'origine cutanée de ces germes est probable. Même extrêmement minutieuse, la désinfection de la peau ne peut éliminer 100% de la flore cutanée.

Pathogénie indirecte

La sécrétion de bêtalactamases par les *Bacteroides* peut protéger les germes sensibles, de l'action des pénicillines. Ce phénomène est observé au cours de nombreuses infections : amygdalites, pharyngites, infections respiratoires (otites chroniques, sinusites chroniques, pneumopathies d'inhalation, abcès pulmonaires), infections pelviennes, infections abdominales, infections cutanées ou des tissus mous. On peut citer de nombreux exemples tels que la protection du streptocoque A de la pénicilline par les bêtalactamases de *Staphylococcus aureus, Haemophilus influenzae, Branhamella catharralis, Bacteroides melaninogenicus, B. fragilis*. De même, les *Bacteroides* protègent *Escherichia coli* dans les infections abdominales et *Neisseria gonorrhoeae* dans les infections pelviennes.

Y. PEAN et Y. BUISSON

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Flow cytometric analysis of within-strain variation in polysaccharide expression by *Bacteroides fragilis* by use of murine monoclonal antibodies

DEBORAH A. LUTTON, SHEILA PATRICK*, A. D. CROCKARD, LINDA D. STEWART†, M. J. LARKIN,† EVELYN DERMOTT and T. A. McNEILL

Department of Microbiology and Immunobiology, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN and †Division of Genetic Engineering, School of Biology and Biochemistry, Queen's University of Belfast, Northern Ireland

Summary. The reactivity of four different monoclonal antibodies (MAbs) with populations of *Bacteroides fragilis* NCTC 9343, enriched by density gradient centrifugation for a large capsule, small capsule and electron-dense layer (EDL) only visible by electronmicroscopy, was examined. The MAbs reacted strongly with polysaccharides present in both the large capsule- and EDL-enriched populations but not in the small capsule-enriched populations. The pattern of labelling was determined by immunoblotting, immunofluorescence and immuno-electronmicroscopy, and flow cytometry. The MAbs labelled cell membrane-associated epitopes in the large capsule- and EDL-enriched populations and cell-free material in the EDL population. By immunoblotting, ladders of repeating polysaccharide subunits were evident in the EDL population but not in the large capsule population. The proportion of cells labelled within each population was determined by flow cytometry. The reactivity of another MAb with the small capsule population was confirmed by flow cytometry. A qualitative indication of epitope expression was obtained by examination of the flow cytometric profiles. Differential expression of the same saccharide epitope was observed both between and within structurally distinct *B. fragilis* populations. The MAbs were species-specific and cross-reacted with several recent clinical isolates. These polysaccharides may be relevant to the virulence of *B. fragilis*.

Introduction

Bacteroides fragilis is a gram-negative anaerobic species that is an important pathogen, commonly encountered in clinical infections.[1] One of the many factors that undoubtedly contribute to its virulence is the expression of particular surface antigens.[2] Several authors have described the encapsulating surface structures of *B. fragilis*[3–7] but, as yet, their relationship to virulence remains unclear. Individual strains of *B. fragilis* have been shown by electronmicroscopy to be heterogeneous with respect to expression of encapsulating surface structures; cells with a large capsule, a small capsule and a marginal electron-dense layer (EDL) may be separated by Percoll density gradient centrifugation.[7] Within-strain antigenic heterogeneity has been shown immunologically with anti-polysaccharide monoclonal antibodies (MAbs).[8,9] One MAb reacts with the small capsule but not with the large capsule or EDL.[9] The surface carbohydrates of *B. fragilis* have been shown by immunochemical analysis to be antigenically complex. Most strains possess four distinct structural elements that can be detected by immunoblotting—a rough type LPS, a common antigen, a smooth LPS which runs as a series of closely spaced bands, and a high M_r antigen which may be capsular polysaccharide.[10] How these components relate to the different capsules is not known. The aim of the present study was to obtain MAbs that reacted with the large capsule or EDL populations and to confirm that these structures are antigenically different from the small capsule. Flow cytometry was used to obtain fast and accurate quantitation of bacterial labelling. The reactivity of the MAbs with recent clinical isolates was also determined.

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* Correspondence should be sent to: Dr S. Patrick.

Materials and methods

Bacterial strains

The strains used in this study were *B. fragilis* NCTC 9343 (National Collection of Type Cultures, Colindale

Avenue, London) and BE3 (Department of Medical Microbiology, Vrije Universiteit, Amsterdam), *B. vulgatus* NCTC 10583, *B. thetaiotaomicron* NCTC 10582, *B. ovatus* ATCC 8483 (American Type Culture Collection), *B. distasonis* ATCC 8503, and *B. gingivalis* W83 (Laval University, Quebec, Canada); *B. fragilis* strains LS1–11, *Escherichia coli* and *Staphylococcus aureus* were recent clinical isolates from the Department of Bacteriology, Craigavon Area Hospital, N. Ireland.

Culture methods

Bacteria were grown in minimal medium defined broth.[11] Cultures were incubated at 37°C in an atmosphere of H_2 10%, N_2 80%, CO_2 10% in an anaerobic cabinet MK III (Don Whitley Scientific Ltd, Shipley, W. Yorks). Identification was confirmed with the API20A system.

Separation and enrichment of bacterial populations

Bacterial populations were enriched for different sizes of encapsulating surface structures by Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient centrifugation as previously described.[7]

Preparation of bacterial outer membranes

Outer membranes were prepared as previously described.[12]

Preparation of polysaccharide

Polysaccharide was extracted by the Proteinase K method.[13]

Production of MAbs

A BALB/c mouse was immunised with whole cells of a *B. fragilis* NCTC 9343 population enriched for expression of the EDL. The mouse was inoculated intraperitoneally with 0·5 ml of a bacterial suspension of 1×10^8 cfu/ml in 0·01 M phosphate-buffered saline, pH 7·4 (PBS). A further inoculation of 0·2 ml was given 4 days before the mouse was killed. The spleen cells from the mouse were fused with P3X 63 Ag8-653 (NS-0/1) mouse myeloma cells by treatment with polyethylene glycol 16000 (Sigma) 50% in RPMI 1640 (Flow Laboratories, Paisley, Scotland) following a modification of the method of Galfre and Milstein.[14] Hybrid cell lines were selected with hypoxanthine-aminopterin-thymidine in RPMI 1640 medium containing myoclone fetal calf serum (Gibco) 20%. Culture supernates were removed and screened by ELISA and immunofluorescence microscopy for IgG to the EDL population of *B. fragilis* NCTC 9343.

Isotyping

The isotype of the MAbs was determined with a mouse MAb isotyping kit (Amersham International plc).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed on vertical slab 8% gels with the Laemmli buffer system.[15] For outer membrane proteins, samples were mixed with 50 µl of PAGE sample buffer (0·0625 M Tris-HCl, pH 6·8, containing SDS 2% w/v, glycerol 10% v/v, 2-mercaptoethanol 5% v/v and bromophenol blue 0·001% w/v) and heated in a boiling water bath for 10 min. After cooling, samples were loaded on to gels. For polysaccharide, proteinase K extracts (50 µl) were loaded on to gels. Gels were run at a constant current of 50 mA/gel. Immunoblotting was performed as previously described.[12]

Culture of bacteria in vivo

Chambers were constructed, filled with *B. fragilis* NCTC 9343 and implanted in the peritoneal cavity of a mouse as previously described.[16] The chambers were constructed with 3-µm pore membrane filters (Millipore) which allowed the entry of leucocytes.

Electronmicroscopy

Bacteria were embedded for ultra-thin sectioning as previously described.[9] For negative staining, one drop of bacterial suspension in distilled water was dried on to a formavar carbon-coated 400-mesh copper grid (Agar Aids) which had been glow-discharged. The grids were labelled with immunogold as previously described.[8] The thin sections were stained with uranyl acetate for 10 min. The whole bacteria were negatively stained with a drop of methylamine tungstate (Agar Aids) 2% w/v in distilled water. The bacteria were examined with a Phillips CM10 transmission electron-microscope.

Dual colour immunofluorescence microscopy

A modification of the staining procedure described by Johnson *et al.*[17] was used. Separated bacterial populations were suspended in PBS, fixed on multitest slides (Flow Laboratories) and incubated with MAb supernates. The slides were washed in PBS then incubated with rabbit anti-*B. fragilis* polyclonal antibody. The slides were again washed and then incubated simulataneously with goat anti-mouse fluorescein conjugate (Sigma) and goat anti-rabbit rhodamine conjugate (Sigma). After a final wash, the slides were examined with a Leitz fluorescence microscope. Random fields of view were selected and the number of bacteria labelled with MAb and polyclonal anti-

serum were counted. The percentage of each population labelled by MAb was calculated relative to the total number of cells labelled with polyclonal antiserum.

Flow cytometric analyses

Cells were prepared by a modification of the method of Nelson *et al.*[18] PBS that had been filtered three times through 0·22-μm membrane filters was used throughout. Washed, late exponential phase cultures of *B. fragilis* were resuspended to approximately 2×10^8 cfu/ml in PBS. Each sample of 1 ml of bacterial suspension was centrifuged at 12 000 *g* in an Eppendorf 5414 centrifuge for 2 min. The pellets were resuspended in 1 ml of MAb supernate or 1 ml of PBS (control) and incubated at 37°C for 1 h. Samples were washed twice in PBS and mixed with 1 ml of appropriately diluted goat anti-mouse IgG fluorescein conjugate (Sigma) or goat anti-mouse IgM fluorescein conjugate (Serotec). The samples were incubated at 37°C for 1 h, washed twice in PBS and resuspended in 1 ml of paraformaldehyde 1% v/v in PBS.

Cells stained with FITC conjugate but without primary antibody were negative controls. An IgM MAb, Bf4, previously shown to react with the small-capsule population[9] was used as a positive control for the fluorescent signal. All samples were sonicated for 20 s at an amplitude of 5 μm (MSE Soniprep 150) to break bacterial aggregates. The sonication time was determined empirically to obtain maximum disruption of bacterial aggregates without cell membrane breakage.

The bacteria were analysed on an EPICS 5 flow cytometer (Coulter Electronics Ltd) equipped with a 5 W argon laser tuned to 488 nm and operating at a power output of 100 mW. Before each analysis, the instrument was calibrated with 1·5-μm fluorescent microspheres (Polysciences). Monodisperse bacterial populations were identified on the basis of size by forward and 90° light scatter signals and gated appropriately. Single parameter log integral green fluorescence (LIGFL) signals were obtained from the gated population. In each sample, 50 000 bacteria were counted and the percentage of positively staining cells was obtained by subtraction of negative control histograms from test histograms with the Immunoprogramme of the instrument.

Results

Characterisation of MAbs and distribution of epitopes in B. fragilis populations enriched for different encapsulating structures

MAb QUBf5. Immunoblotting of proteinase K extracts with MAb QUBf5, of isotype IgG_{2b}, showed a diffuse staining pattern at the top of the blot, corresponding to a high M_r polysaccharide (fig. 1a, b and c). This polymer was present in smaller amounts in the small capsule population (fig. 1b). A series of discrete bands of repeating polysaccharide subunits was found to extend from the high M_r polysaccharide to the gel front in the EDL population (fig. 1c). Electronmicroscopy and immunogold labelling with MAb QUBf5 identified a surface-associated antigen located on the membranes of whole bacteria and on membrane vesicles or blebs. Only a proportion of the bacteria within the large capsule and the EDL populations was labelled (not illustrated). Cells from the small capsule population did not react with MAb QUBf5 but labelled blebs appeared to be secreted from unlabelled cells (fig. 2A). The QUBf5 epitope was readily released from the large capsule population in association with membrane blebs (fig. 2B) and from the EDL population as both membrane blebs and cell-free material (not illustrated).

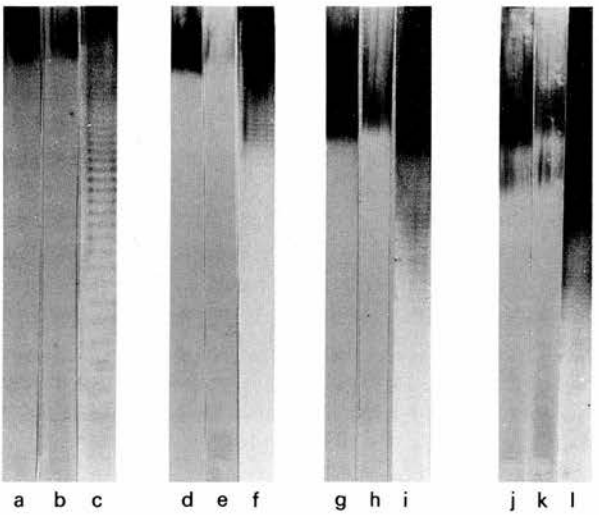

Fig. 1. Immunoblots from SDS-PAGE gels of proteinase K extracts of *B. fragilis* NCTC 9343. Lanes **a**, **d**, **g** and **j**: large capsule population; lanes **b**, **e**, **h** and **k**: small capsule population; lanes **c**, **f**, **i** and **l**: EDL population. The blots were probed with MAbs QUBf5 (lanes **a–c**), QUBf6 (lanes **d–f**), QUBf7 (lanes **g–i**) and QUBf8 (lanes **j–l**).

The budding of vesicles from the outer membrane of *B. fragilis* has been observed (fig. 3A). These vesicles may carry encapsulating material (fig. 3B).

MAbs QUBf6 and QUBf7. Two IgG_1 MAbs, QUBf6 and QUBf7, each reacted with high M_r polysaccharides to give diffuse staining bands at the top of the immunoblots (fig. 1d–i). The quantity of labelled polysaccharide was less in the small capsule population (fig. 1e and h). Associated fine ladders of repeating polysaccharide subunits were detected in the EDL population (fig. 1f and i). Heterogeneous labelling of the large capsule (fig. 4A) and the EDL (not illustrated) populations was detected by electronmicroscopy and immunogold labelling with each MAb. Labelled cells were not seen in the small capsule population with either MAb, although some cell-free material was detected by MAb QUBf7 (fig. 4B). Cell-associated polysaccharides were recognised by each MAb in the large capsule (fig. 4C) and the EDL (fig. 4D) populations as illustrated with MAb QUBf7. These

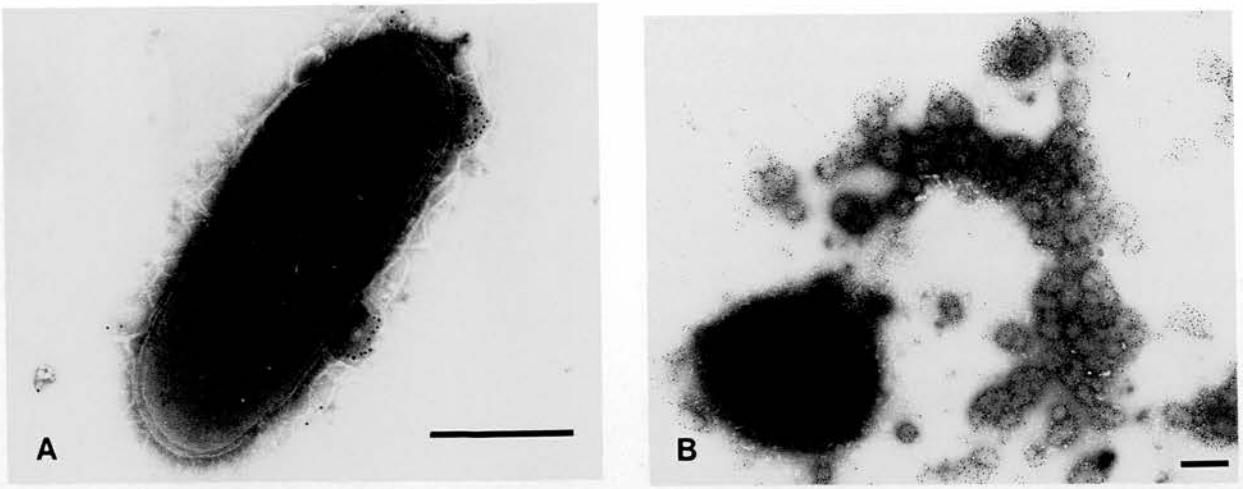

Fig. 2. Negative stained electronmicrographs of (**A**) the small capsule and (**B**) the large capsule populations of *B. fragilis* immunogold labelled with MAb QUBf5. Note the labelling of excreted vesicles. Bar, 0·5 μm.

antigens were released from the large capsule population in association with membrane vesicles (fig. 4C) and from the EDL population as membrane vesicles and cell-free material (fig. 4D).

MAb QUBf8. An IgG_1 MAb, QUBf8, was shown by immunoblotting to recognise a high-M_r polysaccharide which produced a diffuse staining band on the top half of the blot (fig. 1j, k and l). Faint labelling was detected just behind the gel front in the region of the rough LPS. The QUBf8 epitope was most readily detected in the EDL populations where a fine ladder was seen in the middle region of the blot (fig. 1l), As with the other MAbs, the antigen recognised was present in smaller quantities in the small capsule population (fig. 1k). The QUBf8 epitope was shown by thin-section immuno-electronmicroscopy to be expressed on a high proportion of cells from the large capsule and the EDL populations (not illustrated). Negative staining revealed that the polysaccharide was cell associated but could be released from the large capsule population in association with membrane vesicles and from the EDL population as cell-free material (not illustrated).

Immunoblotting of outer membrane extracts

The immunoblotting patterns of Sarkosyl-extracted outer membranes were similar to those obtained by proteinase K extraction (fig. 1). No additional bands were identified in the outer-membrane preparations, indicating that protein antigens were not detected by any of the MAbs.

Confirmation of MAb specificity by immunofluorescence microscopy and flow cytometry

The pattern of labelling of the large capsule, small capsule and EDL populations with the MAbs was confirmed by immunofluorescence microscopy and flow cytometry. Strong labelling was associated with the small capsule populations in the case of MAb Bf4 and with the large capsule and the EDL populations in the case of the other MAbs (table I and fig. 5). The proportion of bacteria labelled within the populations was also measured (table I). For microscopy, populations were labelled at the same time with a rabbit

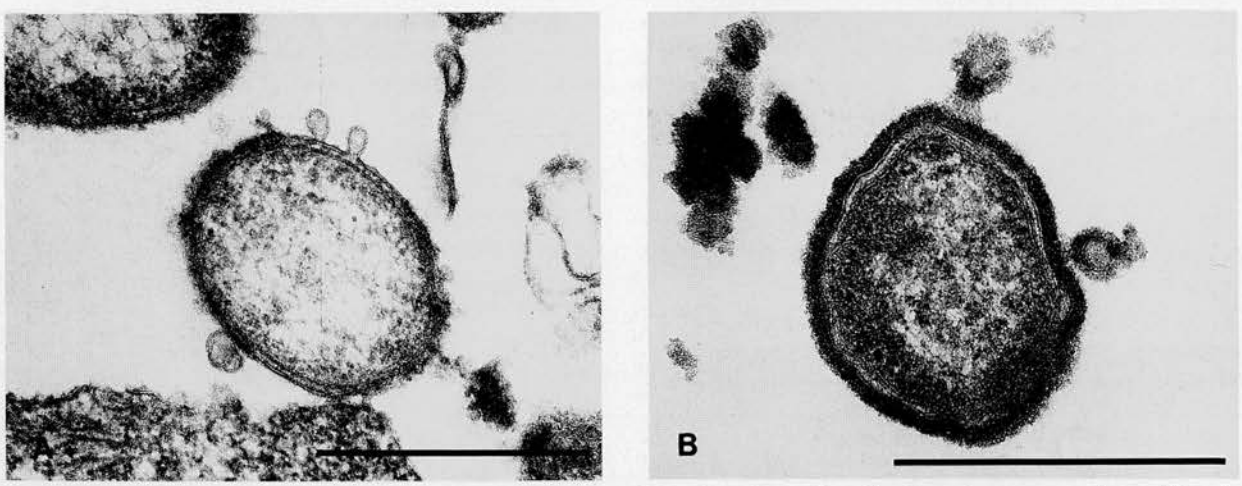

Fig. 3. Thin section electronmicrographs of *B. fragilis* grown *in vivo*. Note the release of vesicles from the outer membrane (**A**) and the association of encapsulating material with released vesicles (**B**).

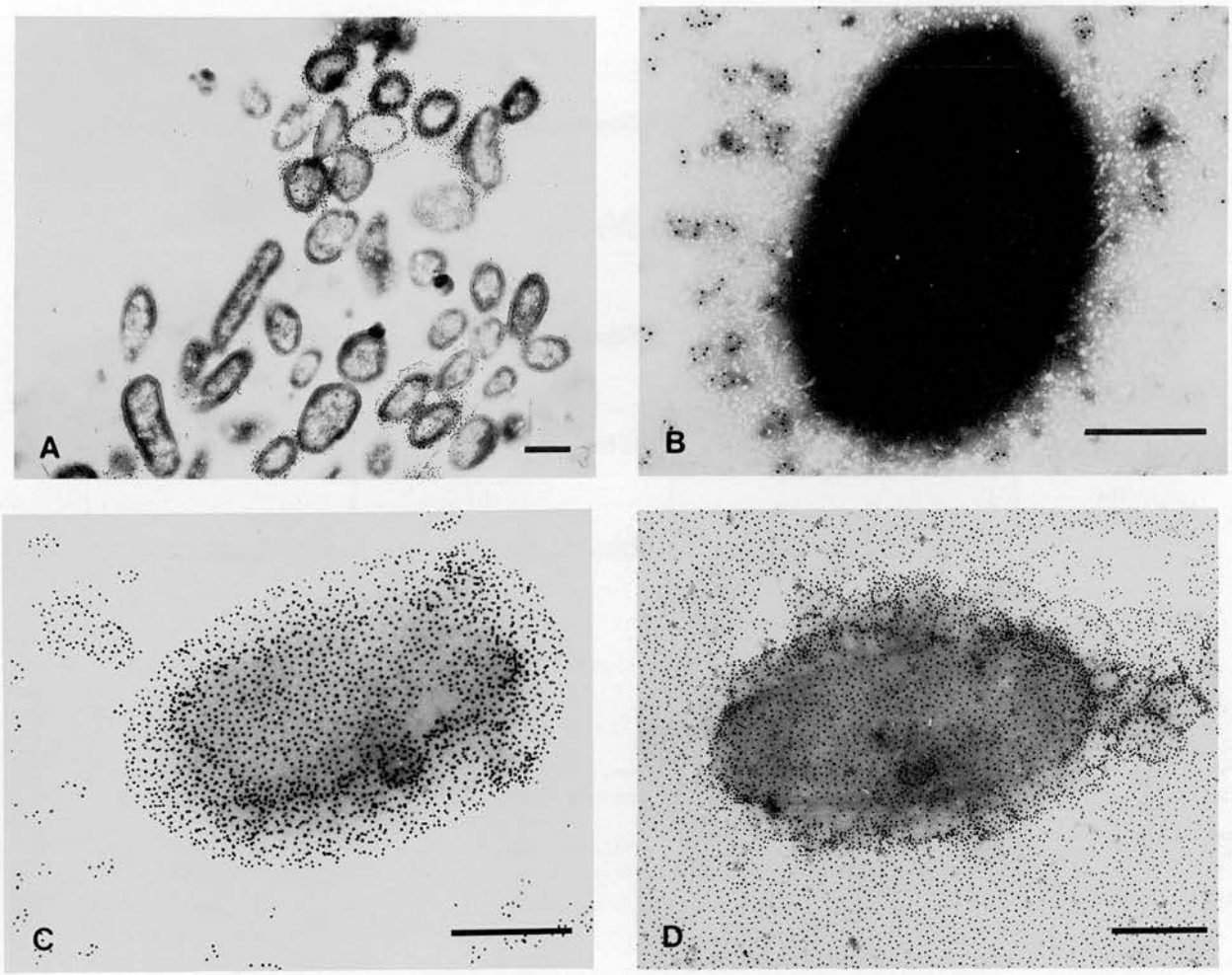

Fig. 4. Electronmicrographs of *B. fragilis* immunogold labelled with MAb QUBf7. (**A**) Ultra-thin section of the large capsule population; note heterogeneous labelling of bacteria. (**B–D**) Negatively stained bacteria: (**B**) small capsule; (**C**) large capsule and (**D**) EDL populations; note cell and vesicle association of labelling with large capsule population and cell free material with EDL population.

polyclonal anti-*B. fragilis* anti-serum (which labelled all the bacteria) and with each of the MAbs. Secondary antibodies of anti-mouse antibody conjugated to fluorescein and anti-rabbit antibody to rhodamine were used to assess the labelling. For flow cytometry, bacteria were identified on the basis of size by forward and 90° light-scatter signals. Bacterial populations labelled with the nucleic acid-specific fluorochrome propidium iodide and identified in the flow cytometer by a red fluorescent signal were identical to those identified by size (unpublished result). Bacteria labelled with the MAbs were identified by the fluorescent

Table I. Variation in MAb labelling in different populations of *B. fragilis* NCTC 9343

MAb	Method of analysis	Mean (SEM) percentage of population labelled*		
		Large capsule	Small capsule	EDL
Bf4	IFM	3 (1)*	96 (5)	0
	FC	9 (5)†	94 (3)	4 (4)
QUBf5	IFM	16 (5)	0	28 (5)
	FC	23 (8)	1 (1)	25 (9)
QUBf6	IFM	14 (5)	0	42 (12)
	FC	37 (5)	7 (7)	47 (14)
QUBf7	IFM	47 (12)	1 (1)	41 (6)
	FC	73 (9)	6 (6)	57 (3)
QUBf8	IFM	19 (14)	0	26 (2)
	FC	77 (11)	5 (4)	67 (11)

IFM, immunofluorescence microscopy; FC, flow cytometry.
* Each IFM value represents the mean of percentages from three experiments.
† Each FC value represents the mean of percentages from three experiments for MAbs QUBf7 and 8 and four experiments for the other MAbs.

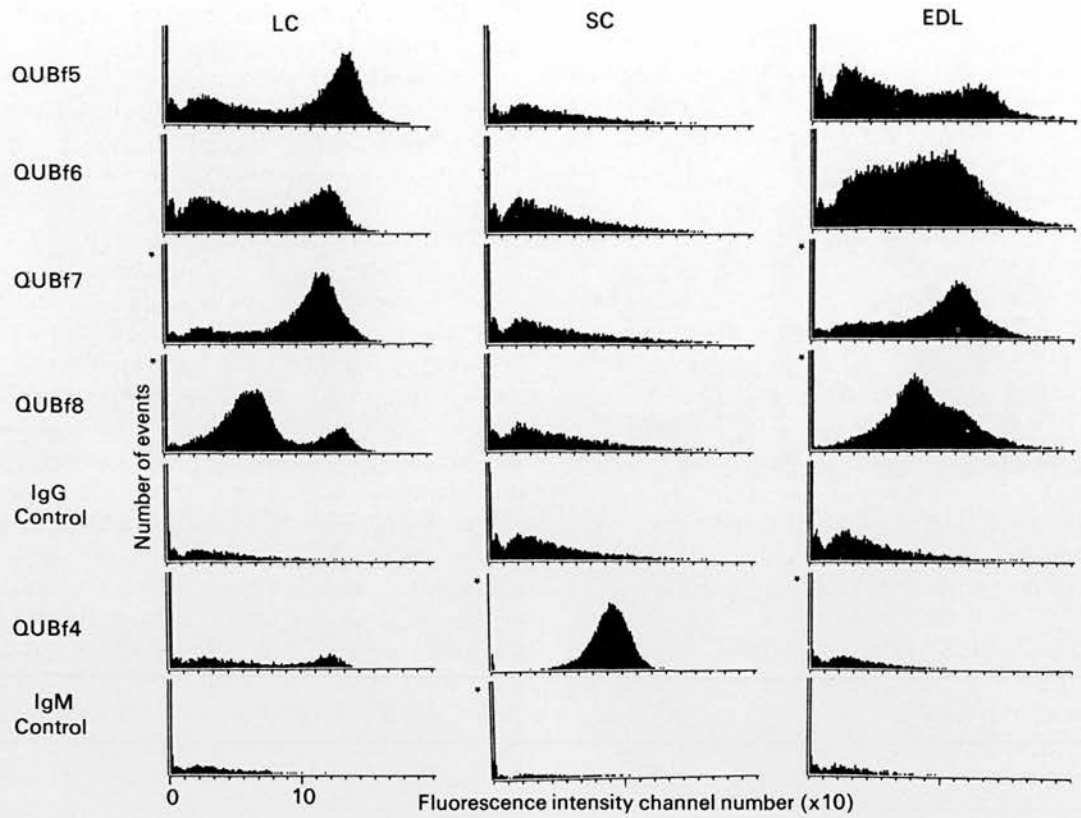

Fig. 5. Flow cytometric profiles. Large capsule (LC), small capsule (SC) and EDL populations of *B. fragilis* were treated with MAbs QUBf5–8 or Bf4 or PBS (control) and the appropriate IgG or IgM fluorescein-conjugated secondary antibodies. Number of events: scale 0–1024 (*), 0–512 for other profiles.

signal from an anti-mouse fluorescein-conjugated secondary antibody. Flow cytometry consistently gave higher percentage labelling than fluorescence microscopy for most of the MAbs.

Flow cytometric profiles, which relate to the intensity of fluorescent signal, were obtained consistently in consecutive experiments. Representative profiles are illustrated in fig. 5. MAb QUBf8, labelling the large capsule population, was an exception; although a biphasic profile was consistently obtained, the relative sizes of the two peaks varied. MAbs QUBf5, 6 and 8 all had a biphasic profile when labelling the large capsule population and a much broader profile with the EDL population. A single peak was obtained with MAb QUBf7 for both the LC and EDL populations, and with MAb Bf4 for the small capsule population. A small peak at high fluorescent intensity was also apparent in the large capsule population with MAb Bf4. A low level of fluorescent signal was obtained with MAbs 5–8 and the small capsule population.

Cross-reaction of the MAbs with other isolates

The reactivity of MAbs QUBf5, 6, 7 and 8 with other *Bacteroides* spp. and with *E. coli* was tested by immunofluorescence microscopy. None of the MAbs cross-reacted with *B. vulgatus, B. thetaiotaomicron, B. ovatus, B. distasonis, B. gingivalis, E. coli* or *S. aureus*. All reacted with *B. fragilis* strain BE3. The specificity of MAb Bf4 was described previously.[9] Several recent clinical isolates, LS1–11, were screened for reactivity with each of the MAbs. The results are presented in table II.

Populations of *B. fragilis* NCTC 9343 grown *in vivo* in the mouse for 7 days showed heterogeneous immunogold labelling with MAbs QUBf7 and 8 (not illustrated).

Discussion

The exact nature of the polysaccharides produced by *B. fragilis* is not yet clear. LPS of *B. fragilis* was characterised chemically and immunochemically by Kasper *et al.*[19] and Weintraub *et al.*[20] The extraction

Table II. Reactivity of *B. fragilis* MAbs with a range of recent clinical isolates of *B. fragilis*

MAb	Number of isolates* which showed positive fluorescence with MAb
Bf4	11
QUBf5	5
QUBf6	4
QUBf7	6
QUBf8	5

* A total of 11 isolates (LS1–11) was screened with each MAb.

and separation procedures used in both studies resulted in the selection of only the rough form of *B. fragilis* LPS. Further assays by gel electrophoresis of the crude aqueous phase obtained by phenol-water extraction or the PCP-insoluble fraction repeatedly failed to detect the ladder-like pattern characteristic of O-antigen.[22] However, Poxton and Brown[10] extracted LPS from *B. fragilis* by the classical aqueous phenol technique and demonstrated that, in most strains, LPS was present as predominantly smooth type. SDS-PAGE analysis revealed regularly spaced ladder patterns of smooth LPS molecules possessing increasing numbers of O-antigen repeating units. Linko-Kettunun *et al.* produced MAbs to the LPS of *B. fragilis*.[22] Each MAb recognised a common galactose-containing determinant present in the outer core of the rough LPS.[23] The absence of the long chains of repeating polysaccharide units led the authors to conclude that *B. fragilis* LPS resembled the rough type LPS of gram-negative bacteria.[24]

The MAbs described in the present study all reacted with saccharide epitopes. By immunoblotting, MAb QUBf5 reacted with *B. fragilis* antigens to produce a ladder in the middle of the blot similar to the ladder which Poxton and Brown suggested was the O-antigen.[10] MAbs QUBf6 and QUBf7 were specific for different high-M_r polysaccharides. MAb QUBf8 labelled high M_r polysaccharide and also some low M_r material which stained in a similar position to the core region of the rough LPS from other bacteria. This indicates that the Bf8 epitope may be present on a core or anchoring region of the polysaccharide.

Therefore, by using whole bacteria as antigens for both immunisation and MAb screening, we obtained a broader range of MAbs, including one which may be to the O-antigen.

Immuno-electronmicroscopy showed that these MAbs labelled epitopes primarily associated with the large capsule and EDL, but not the small capsule. In the case of the large capsule population, MAbs QUBf5–8 recognised epitopes present on both the bacterial surface and extracellular vesicles. The membrane vesicles either remained within the fibrous network of the large capsule or were sloughed off. Since extracellular vesicles may have associated capsular or EDL material, it remains unclear whether the saccharide epitopes are associated with the membrane bilayer or the encapsulating structures. Deslauriers *et al.* observed the release of similar extracellular vesicles from intact *Porphyromonas (Bacteroides) gingivalis* cells.[25] The extracellular vesicles described consisted of a membrane bilayer and were thought to contain some periplasmic material. The release of extracellular OM vesicles has previously been described in *E. coli* growing under lysine limitation[26] and also under normal growth conditions.[27] Such a release of membrane vesicles or slime may be important in virulence. The antigenic sites located on extracellular structures could result in the deposition of opsonin, either C3 or specific antibody, at a distance from the bacterial cell, thus reducing the effectiveness of the host immune response.

The reaction of each of the anti-polysaccharide MAbs with a proportion of recent clinical isolates and bacteria grown *in vivo* suggests that these polysaccharides may be relevant to the virulence of *B. fragilis*.

In contrast with the large capsule population, in the EDL population the saccharide epitopes were not firmly surface-associated. Polysaccharides are abundantly released from these cells as free material, probably in the form of slime (fig. 4d) similar to that observed in other bacteria and described as excreted viscous substances that are not anchored in the bacterial outer membrane.[28]

Interestingly, the ladder pattern observed by immunoblotting was associated only with the EDL population. These ladders are considered to represent polysaccharide molecules of varying chain lengths, each step up the ladder corresponding to the addition of one repeating subunit. Perhaps the polysaccharides from cells in the EDL population are of varying chain lengths, or they are more susceptible to breakage of the chain. Where the polysaccharides remain solely cell- or vesicle-associated, in the case of the large capsule population, the chains may remain large and intact.

There was a low level of labelling of the small capsule population detectable by flow cytometry, immunoblotting and immunogold electronmicroscopy with negative stain. There are a number of possible explanations of this. Firstly, as the MAb only labelled extracted and secreted material and not whole cells, it is possible that the antigenic sites are masked by other structures. However, electronmicroscopy and immunogold labelling of thin sections gave a negative result and, therefore, did not support this hypothesis. Secondly, a low proportion of cells from the large capsule or EDL population may have been present in the sample. Thirdly, the polysaccharides may be expressed at a low level because of incomplete repression of the genes involved in controlling polysaccharide synthesis. A few labelled outer membrane vesicles were observed in the small capsule population (fig. 2a).

Flow cytometry provided further confirmation of the labelling pattern of the four MAbs QUBf5–8 and of MAb Bf4 which reacted with the small capsule. It also allowed a more rapid and accurate measurement of the proportion of bacteria labelled within a given population.

The generally higher percentage labelling obtained by flow cytometry, when compared with fluorescence microscopy, may be due to the greater sensitivity of the flow cytometer which detects fluorescence signals not readily visible by eye. Alternatively, it may be due to the harsher fixation procedure used for microscopy. This may cause the loss of some antigenic sites.

The significance of the fluorescent intensity profiles obtained by flow cytometry is at yet unclear, although they were consistently reproducible. The twin peaks

obtained with MAbs 5, 6 and 8 and the large capsule population may represent different populations within the large capsule population. Whether this occurs as a natural pheonomenon or as a result of the treatment of bacteria during the labelling procedure is under investigation. These MAbs gave a broad profile when labelling the EDL population. The electronmicroscopy results show that polysaccharides labelled by these MAbs are released as cell-free material from the EDL population. Therefore, the broad profile may represent a continuum from cells from which most of the polysaccharide has been released as free material, to those in which most of it remains associated with the bacterial cell. Ørskov and Ørskov[30] noted that even the mildest extraction procedures, such as cautious suspension of *E. coli* in saline, gives a suspension containing capsular material and LPS. More discrete peaks (e.g., small capsule population with MAb Bf4) may relate to the closer association of the polysaccharide with the cell surface.

We have shown previously that the EDL population agglutinates erythrocytes from a number of different animal species.[29] The present study shows that a proportion of the bacteria within these populations share common epitopes. The polysaccharides recognised by the MAbs may not be involved in haemagglutination. Alternatively, the polysaccharides may have different properties in the large capsule and EDL populations. This might relate in some way to the variation in polysaccharide chain length detected by immunoblotting only in the EDL population.

The factors governing the differential expression of the same saccharide epitopes in the large capsule, the small capsule and the EDL populations of *B. fragilis* are unclear. Ørskov and Ørskov described form variation in *E. coli* K1 strains.[28] In the same K1 antigen cultures two types of colony were found with different reactivity towards a K1 antiserum. It was suggested that antigenic phase variation occurred in *E. coli* O26/B6.[30] Two distinct phenotypic variants, which showed differences in reactivity with a core-specific MAb, were identified by flow cytometry. Similar antigenic variation may occur in *B. fragilis* and may be important in the virulence of this bacterium.

Structural differences, observed by electronmicroscopy, within individual strains of *B. fragilis* are reflected by differences in antigenicity. There is also, antigenic variation within structurally homogeneous populations of *B. fragilis*. Therefore, it seems likely that antigenic variation may occur in *B. fragilis* and the epitopes expressed by individual cells may reflect the selective pressures imposed on the population at a given time. The stability of epitope expression in relation to the virulence of *B. fragilis* is the subject of current investigations.

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The assessment of faecal flora in patients with inflammatory bowel disease by a simplified bacteriological technique

M. H. GIAFFER, C. D. HOLDSWORTH* and B. I. DUERDEN†

Gastroenterology Unit, Royal Hallamshire Hospital, Sheffield S10 2JF and †Department of Experimental and Clinical Microbiology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

Summary. A semi-quantitative bacteriological method was used to study faecal flora in 42 patients with Crohn's disease, 37 with ulcerative colitis and 21 healthy controls. Faecal homogenates were plated on primary isolation plates by a technique that allowed the growth of various microbial isolates to be assessed on a visual 1+–5+ score. This method was first calibrated against a standard quantitative bacteriological technique, which confirmed the reliability and reproducibility of the results obtained by the simpler method. Patients with clinically active Crohn's disease (22) had significantly higher total aerobe scores than patients with quiescent disease (20) ($p \leq 0{\cdot}006$) or ulcerative colitis ($p \leq 0{\cdot}04$) or normal controls ($p \leq 0{\cdot}02$). The scores of *Escherichia coli* were parallel to those of total aerobes. Lactobacillus and bifidobacteria scores were significantly reduced in patients with Crohn's disease compared to those with ulcerative colitis and controls. The anaerobic flora in both Crohn's disease and ulcerative colitis was indistinguishable from that of controls. *Bacteroides vulgatus* and *B. fragilis* were the predominant bacteroides in all groups. Patients with ulcerative colitis, regardless of disease activity, harboured faecal flora that did not differ from that of normal controls. The abnormal faecal flora in Crohn's disease did not correlate with established clinical and laboratory indicators of disease activity.

Introduction

The quantitative surface viable count technique described by Miles *et al.*[1] is the only common bacteriological method applied for the enumeration of the various components of the faecal microbial flora. It has the disadvantage of being laborious and time-consuming and may influence the number of faecal samples that can be analysed in a busy laboratory. Direct microscopic counting of the faecal microbes by microscopy has also been used to evaluate the composition of faecal flora but this does not differentiate between dead and viable bacteria and studies comparing the two bacteriological methods have frequently given conflicting results.[2] Neither method can be reproduced with accuracy; direct microscopic counting is the least reliable. For the analysis of a large number of faecal samples, the availability of a simple, reliable and reasonably accurate bacteriological method is desirable.

A semi-quantitative microbiological method is described that can be used for the assessment of faecal microbial flora in health and disease. This gives results comparable to those obtained by the standard surface viable count, a technique which has been widely used to enumerate bacteria in faecal specimens.[3–6] The results of the application of this method to determine the composition of the faecal flora in patients with inflammatory bowel disease are also presented.

Materials and methods

Patients

Twenty-nine patients with inflammatory bowel disease and 21 healthy individuals were studied. In all patients the disease was diagnosed by standard clinical, radiological and histological criteria.[7]

Forty-two patients had Crohn's disease. In 22 of these (16 females and 6 males; mean age 38 years), the disease was considered to be active. Disease activity was assessed by the presence of significant symptoms, (e.g., diarrhoea, abdominal pain, weight loss), abnormal laboratory tests generally accepted as reflecting active gut inflammation[8] and a Crohn's Disease Activity Index (CDAI) of >150.[9] Five patients had

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* Correspondence should be sent to Dr C. D. Holdsworth.
† Present address: Department of Medical Microbiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN.

Patrick S.
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Addendum

Cases with unexplained severe immunosuppression but without evidence of HIV infection have been described. A recent expert meeting of the World Health Organization concluded, however, that they are not epidemic in nature, they may represent different entities with various causes and that at present there is no evidence that such a condition would be transmitted by blood.[48]

REVIEWS IN MEDICAL MICROBIOLOGY

The virulence of *Bacteroides fragilis*

S. Patrick

Department of Microbiology and Immunobiology, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK

Summary. The obligately anaerobic Gram-negative intestinal bacterium *Bacteroides fragilis* is a frequent cause of sepsis arising from faecal contamination. The precise nature of the virulence determinants of this bacterium remain to be elucidated: polysaccharides, fimbriae, extracellular enzymes and enterotoxin may all play a role. It is likely that surface structure variation is also of prime importance to virulence.

Introduction

The Gram-negative bacterium *Bacteroides fragilis* is the obligate anaerobe most frequently isolated from clinical infection. Sources include intra-abdominal, vaginal, pilonidal, perianal, brain and lung abscesses. Recently *B. fragilis* was reported to be present in 80–90% of cases of peritonitis and *Escherichia coli* in approximately 60% of cases.[1]

Such infections generally arise from faecal contamination. In the case of intra-abdominal infection this may be the result of, for example, a ruptured appendix. Faeces normally contain between 10^{11} and 10^{12} bacteria per g, which in terms of volume of the bacteria, leaves little room for anything else. The anaerobic bacteria within faeces outnumber the facultative bacteria, such as *E. coli* by about 1000 to 1. Thus, anaerobic bacteria constitute approximately 99% of the faecal bacterial mass. Of this, *Bacteroides* spp account for 20–30% of the species isolated.[2] The *Bacteroides* spp *fragilis, distasonis, vulgatus, ovatus, thetaiotaomicron, caccae, eggerthii, merdae, stercoris* and *uniformis* constitute the 'fragilis group' of bacteroides with *B. fragilis* as the type species. Prior to 1976,[3] *B. fragilis, distasonis, ovatus, thetaiotaomicron* and *vulgatus* were designated subspecies of *B. fragilis*, e.g. *B. fragilis* subsp *vulgatus*. It is therefore important to ascertain, when reading the literature, whether the authors are referring to *B. fragilis*, the species, or *B. fragilis* as a group including the former subspecies.

As anaerobic bacteria predominate in faeces, the association of these bacteria with infections resulting from faecal contamination of body sites is, perhaps, not surprising. If, however, the composition of the anaerobes in faeces is examined, the most abundant species is *B. vulgatus*, with *B. fragilis*, the most common clinical isolate, only a relatively minor component.[4] More recent studies indicate that the occurrence of *B. fragilis* in the intestinal flora has been underestimated. Namavar and colleagues[5] investigated the colonic, rather than the faecal flora, by faecal lavage and biopsy of colonic mucosa. They estimated the proportion of *B. fragilis* in the colonic flora was 44%, in comparison with approximately 4% in the faecal flora. *B. vulgatus* accounted for 45% of the faecal flora and about 30% of the colonic flora.

Possible virulence determinants of *B. fragilis* include encapsulating surface structures outside the outer membrane, filamentous fimbriae, the release of factor(s) which inhibit phagocytic function, the release of extracellular degradative enzymes and enterotoxin.[6] There are varying degrees of evidence for the role these determinants play in virulence, but it is likely that they all play some part, maybe at different stages in the pathogenic process. This review is intended to be a personal view of the current state of understanding (or lack of) of some of the virulence determinants of *B. fragilis*. A discussion of enterotoxin production by *B. fragilis* and its role in diarrhoea is beyond the scope of this review. The reader is referred to the publications of Myers and colleagues.[7]

Virulence: structures

A confusion of polysaccharides

Studies of the virulence determinants of *B. fragilis* have been retarded by a general acceptance that, firstly, any population grown under constant conditions is homogeneous with respect to surface structure expression and that, secondly, the presence of a polysaccharide capsule is a unique feature of *B. fragilis* which is lacking in other *Bacteroides* spp and therefore the major virulence determinant. A crude polysaccharide extract from strain ATCC 23745, which contains a mixture of polysaccharides,[8] will cause sterile abscess formation in animal models of infection.[9] Immunization with this polysaccharide mixture protected against abscess formation;[10] however, this is far from the complete story of *B. fragilis* virulence, or indeed *B. fragilis* surface polysaccharides.

Evidence for within-strain variation in *B. fragilis* capsule expression and for expression of capsules in other species of the fragilis group was published by Babb & Cummins in 1978.[11] These authors observed capsules of different sizes within individual strains of *B. fragilis* by light microscopy of wet India ink preparations. Evidence of possible within-strain antigenic variation was obtained indirectly by Schwann & Danielsson[12] who observed non-homogeneous staining of *B. fragilis* by immunofluorescence microscopy using polyclonal antisera. In 1981 we attempted to repeat the observations of Babb & Cummins. We changed the growth medium to determine if this would alter capsule size, as nutrient availability had been reported to alter capsule expression in other bacteria.[13] A change from the peptone yeast extract medium of Duerden and colleagues[14] to the minimal medium of van Tassel & Wilkins[15] resulted in greatly enlarged capsules as observed by light microscopy. This allowed both the accurate quantification of the percentage of bacteria within a population bearing large capsules and the separation of these cells.[16] Late exponential phase culture was layered onto a step density Percoll gradient with steps of 80, 60, 40 and 20% Percoll (Fig. 1: method detailed in [17]). After centrifugation, material from the gradient interfaces was sub-cultured into fresh broth, grown and applied to a gradient a second time (Fig. 1Ci–iii). It was now apparent that the gradient centrifugation had enriched for different sub-populations from within one strain of *B. fragilis*. Light microscopy revealed that the 0–20% interface enriched for large capsule (LC) bacteria (Fig. 1Di), the 20–40% interface for small capsule (SC) bacteria (Fig. 1Dii), the 40–60% for a mixture of SC and non-capsulate (NC) bacteria and the 60–80% interface for only NC bacteria (Fig. 1Diii). A number of strains were examined using this technique. Not all strains had the small capsule sub-population (notably strain ATCC 23745) and in some strains a few bacteria passed through the 80% Percoll to the bottom of the centrifuge tube.

Ultrathin section and electron microscopy revealed a large fibrous network in the LC population (Fig. 1Ei) and a smaller fibrous network in the SC population (Fig. 1Eii). Ruthenium red enhanced the electron density of both of these structures when observed by electron microscopy. This suggests that the major components of the LC and the SC are negatively charged polysaccharides.

The population which was NC by light microscopy had a narrow electron dense layer outwith the outer membrane (Fig. 1Eiii). This was termed the electron dense layer (EDL) as it was electron dense (i.e. dark) by electron microscopy *without* ruthenium red stain. This strongly suggests that it has components other than polysaccharides. A few bacteria within the 60–80% enriched population lacked any visible structure outwith the outer membrane. Interestingly NC *B. ovatus* lacked an electron dense layer.

Subsequent studies with monoclonal antibodies (MAbs) using immunofluorescence microscopy, immunoelectron microscopy, immunoblotting and flow cyometry revealed, surprisingly, that the SC was not simply a smaller amount of the LC, but carried antigenically distinct epitopes[18] (Fig. 2). These studies also revealed that the EDL was antigenically distinct from the SC and not simply condensed fibrous material. Immunoblotting after polyacrylamide gel electrophoresis (PAGE) confirmed that the epitope associated with the small capsule was present on a high molecular weight polysaccharide. This, along with the reactivity of another MAb,[19] implied that there were at least two antigenically distinct polysaccharides produced by individual strains of *B. fragilis*.

Subsequently we produced other MAbs which unexpectedly reacted with epitopes in *both* the EDL and LC population[20,21] (Fig. 2). In the LC population the polysaccharide epitopes remained either associated with the cell surface or excreted vesicles of outer membrane (extracellular vesicles; ECV), whereas in the EDL-enriched population the epitopes were associated with copious quantities of excreted extracellular polysaccharide as well as ECV. Immuno-labelling for fluorescence and electron microscopy and flow cytometric analyses revealed a heterogeneous labelling pattern with the MAbs, i.e. only a proportion of the bacteria within the LC or EDL population were labelled with a given MAb[22,23] (Fig. 3). It is likely that this reflects antigenic variation of individual structures. In *E. coli*, within-strain variation in epitope expression has been reported for *E. coli* K18 and K1. *E. coli* K18 and K22 produce capsules which are both composed of polyribosyl-ribitol phosphate. In *E. coli* K18, however, the ribose is O-acetylated and this is sufficient to alter the antigenicity of the molecule. Using antisera specific for these two serotypes and immuno-electron microscopy, it was clear that *E. coli* K18 is in fact a mixture of bacteria, 40% of which express the acetylated form of the capsule, the remainder expressing the non-acetylated form. In *E. coli* K1, the polysialic acid capsule

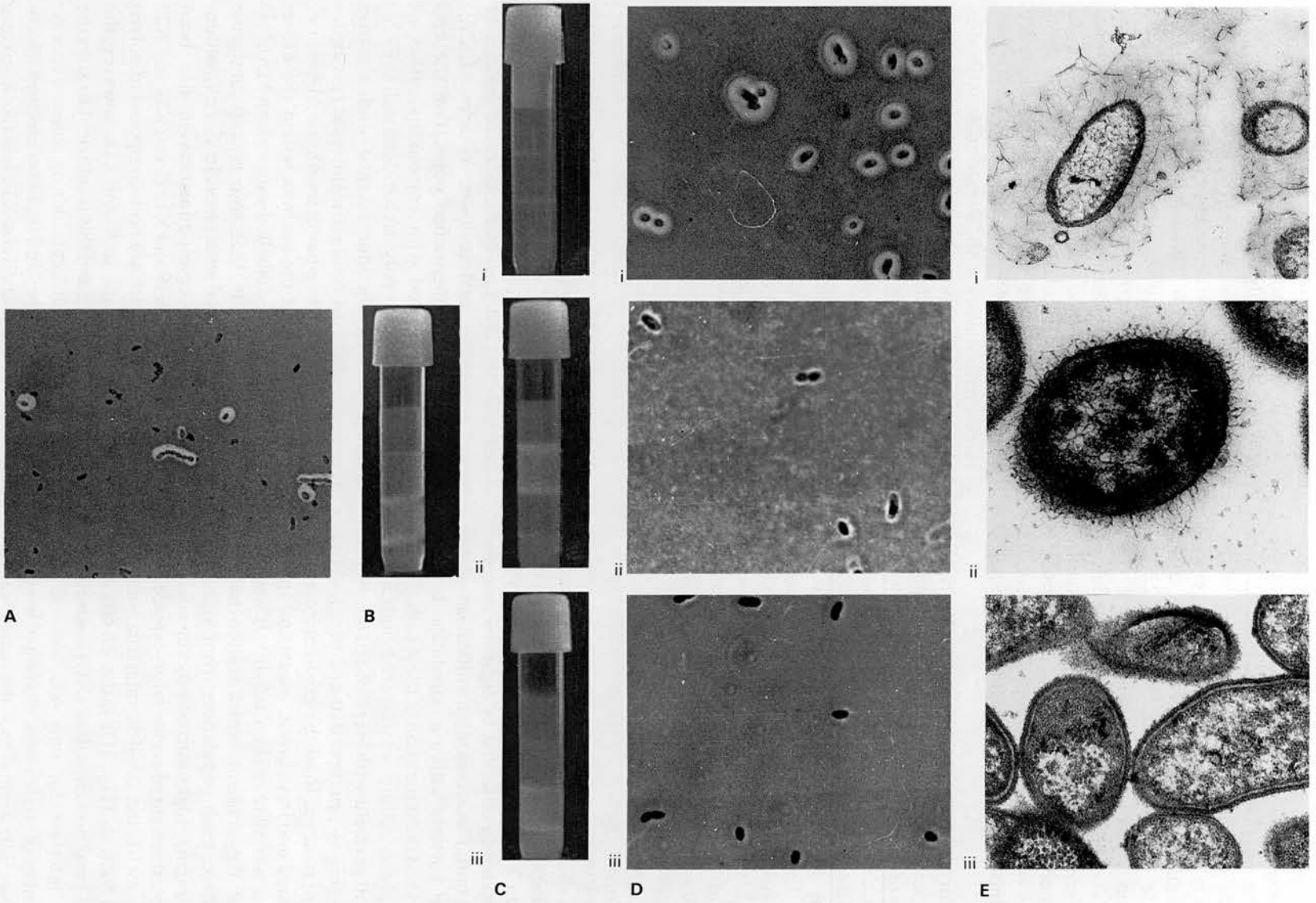

Fig. 1 A. Capsule smear of *Bacteroides fragilis* NCTC 9343 prior to enrichment on a Percoll density gradient. Note large capsule, small capsule and non-capsulate bacteria.
B. *B. fragilis* population as in (A) after centrifugation on a Percoll step density gradient (steps of 20, 40, 60 and 80% Percoll).
C. Sub-culture from the gradient interface layers in (B): (i) 0–20% interface; (ii) 20–40% interface; (iii) 60–80% interface.
D. (i–iii) Capsule smears of populations sampled from the interface layers.
E. (i–iii) Electron micrographs of ruthenium red stained ultra-thin sections of populations sampled from the interface layers.

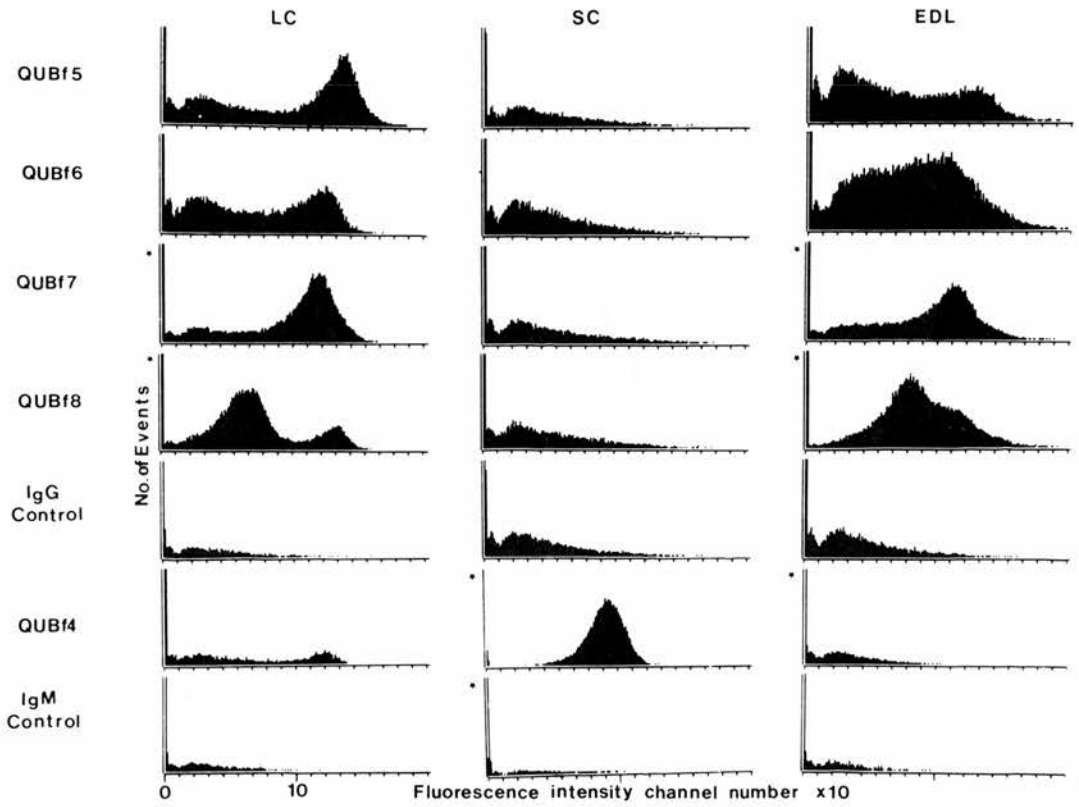

Fig. 2 Flow cytometric profiles of large capsule (LC), small capsule (SC) and EDL populations of *B. fragilis* treated with MAbs QUBf5–8, BF4 or PBS (control) followed by the appropriate IgG or IgM fluorescein-conjugated secondary antibodies. Number of events: scale 0–124 (*), 0–512 for other profiles. Note that Bf4 is specific for the SC population and that the other MAbs label the LC and EDL populations. Reproduced, with permission, from Lutton DA, Patrick S, Crockard AD et al, J Med Microbiol 1991; 35: 229–237.

also exhibits within-strain variation, the $K1^+$ antigen is O-acetylated and slightly immunogenic, whereas the $K1^-$, non-acetylated form is non-immunogenic. It is thought that the variation is mediated by a genetic switch which controls the activity of a specific transacetylase enzyme.[24] Whether similar mechanisms are responsible for the within-strain antigenic variation observed in *B. fragilis* is a matter for future investigation.

In a recent publication,[25] the authors expressed surprise at detecting two different polysaccharides in *B. fragilis* strain NCTC9343. They suggest that one of the two polysaccharides may be the same as the polysaccharide detected by us in the SC population. Unfortunately they did not define the types of capsule expressed within their populations by microscopy, therefore it is difficult to draw any firm conclusions.

This tremendous capacity for within-strain structural and antigenic variation of polysaccharide expression by *B. fragilis* must be taken into account when reading any published literature where the surface structures of the populations under investigation have not been defined by both light and electron microscopy.

Chemical and immunochemical analyses of the surface polysaccharides have yielded apparently contradictory results. *B. fragilis* has been reported to have a 'strain specific' high molecular weight polysaccharide and a largely 'species specific' rough-type lipopolysaccharide (LPS).[8] The LPS was reported to have a core sugar region and short O-antigen, but no repeating long chains of polysaccharide linked to the lipid A. Other authors report a more complex mixture of surface carbohydrates.[26,27] By PAGE and immunoblotting, Poxton & Brown[28] investigated further the purified rough-LPS and capsular polysaccharide fractions obtained after Sephacryl S-300 separation of aqueous phenol extracts by the method of Kasper and colleagues.[8] The fraction containing the high molecular mass polysaccharide also contained smooth LPS, which formed a characteristic ladder pattern on PAGE; the rough-LPS fraction also contained a low molecular weight common polysaccharide antigen which migrates behind the rough LPS. The low molecular weight polysaccharide was common to all the *B. fragilis* strains examined. (Note: This work is misquoted by Lindberg and colleagues,[29] the 'common' polysaccharide antigen does not form a ladder pattern.) The inability by some workers (e.g.[30]) to detect the repeating polysaccharide chains of smooth-type LPS is undoubtedly due, in part, to the use of a phenol-chloroform-petroleum ether (PCP) extraction procedure which will selectively isolate rough-type LPS. The material remaining

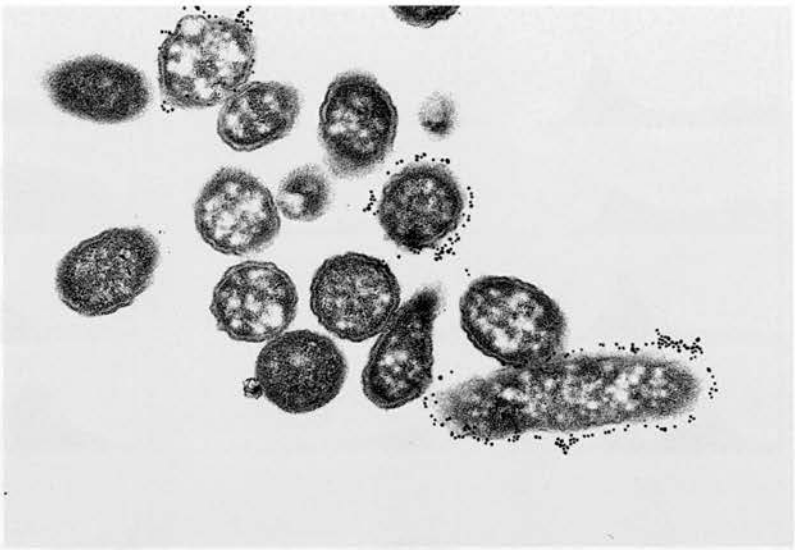

Fig. 3 Ultra-thin section of *B. fragilis* treated with MAb followed by the appropriate gold-conjugated secondary antibody. Note that only a proportion of the bacteria are labelled.

insoluble after the PCP extraction, and normally discarded, contains the *B. fragilis* smooth LPS, common polysaccharide antigen and higher molecular weight polysaccharide.[28]

Different proportions of LC, SC and EDL within the populations used for the extractions will also have a major influence on the polysaccharides detected. We have produced a MAb which detects a ladder pattern, characteristics of smooth-LPS, in immunoblots. The ladder pattern was only detectable on extracts taken from the EDL-enriched population, and not the LC population. This was also the case for the more closely spaced ladder patterns associated with the high molecular weight polysaccharides[20] (Fig. 4).

Interestingly, the EDL population releases the polysaccharide as cell-free slime. Whether the cell-free polysaccharide is present in chains of different lengths, while the cell-associated capsular polysaccharide is of more uniform chain-length, remains to be seen. Alternatively, the cell-free polysaccharide may be more susceptible to chain-breakage.

These results highlight the complexity of *B. fragilis* polysaccharides and underline the need to take into account within-strain variations.

Reports of sugar analysis of the LPS of *B. fragilis* include the following sugars: glucose, galactose, fucose, rhamnose, xylose, glucosamine, galactosamine, ribose, arabinose, mannose (reviewed in[29]). Studies of the rough-type LPS detected the rhamnose, galactose, glucose, galactosamine and glucosamine. On the basis of the distribution of these sugars, 17 strains of *B. fragilis* could be placed into 5 different groups. This core region of LPS could be detected with a MAb specific for the epitope β-1-6 linked galactose disaccharide.[31,32] The authors termed this a common antigenic determinant because the MAb reacted with 96% of strains examined; however, only an estimated 10% of bacteria within a population were labelled after immunogold labelling of ultra-thin sections. It was suggested that the low level of labelling might be related to the growth phase[31] or the harshness of the washing procedures used during the labelling procedure.[32] It is more

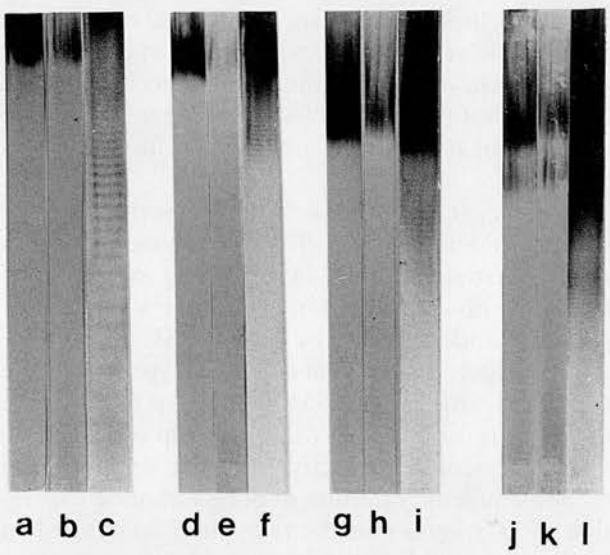

Fig. 4 Immunoblots from SDS PAGE gels of proteinase K extracts of *B. fragilis* NCTC 9343. Lanes a, d, g and j: large capsule population; lanes b, e, h, and k: small capsule population; lanes c, f, i, and l: EDL population. The blots were probed with MAbs QUBf5 (lanes a–c), QUBf6 (lanes D–f), QUBf7 (lane g–i) and QUBf8 (lanes j–l). Reproduced, with permission, from Lutton DA, Patrick S, Crockard AD et al, J Med Microbiol 1991; 35: 229–237.

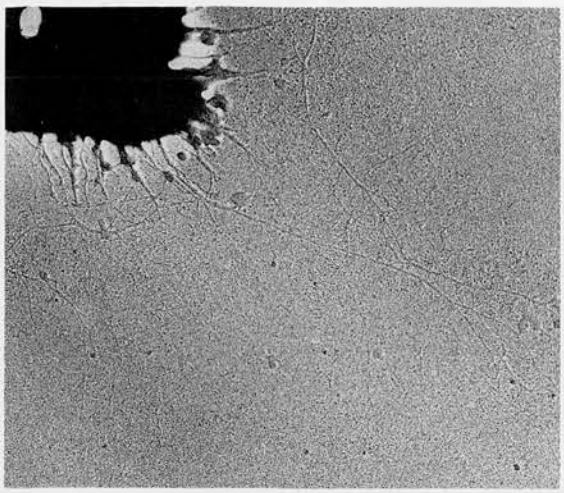

Fig. 5 Electron micrograph of platinum-gold coated whole cells of *B. fragilis*. Note the long strands of material extending from the bacteria.

likely that the low percentage labelling reflects antigenic heterogeneity within the population[22] and this determinant cannot, therefore, be considered to be a 'common' antigenic determinant.

There have been conflicting reports of the presence/absence of L-glycero-D-manno-heptose and keto-deoxyoctonate(KDO), both of which are present in enterobacterial LPS. KDO is almost certainly present, but in a phosphorylated form, rendering it undetectable in the standard assay.[33]

The relationship of polysaccharides to virulence is considered below.

Lipid A

Inter-strain variation in the fatty acid content of the lipid A of *B. fragilis* has been reported (reviewed in[29]). In the lipids examined, only trace amounts of the predominant fatty acids associated with enterobacterial LPS (e.g. 3-hydroxytetradecanoic acid) were detected. The other major differences between *B. fragilis* and enterobacterial LPS were (a) in the number of fatty acids per lipid A (5 for *B. fragilis*, 6–7 for enterobacteria), (b) fatty acid chain length (C15–17 for *B. fragilis*, C12–16 for enterobacteria), (c) branching hydroxylated and non-hydroxylated fatty acids in *B. fragilis* and (d) a lack of 4′ phosphoryl group on the non-reducing glucosamine residue in *B. fragilis*. The biological activities of *B. fragilis* LPS are discussed below.

It remains to be seen if there are within-strain differences in the lipid A content of *B. fragilis* and if the LC, SC and EDL subpopulations are different in this respect.

Fimbriae

Studies of the fimbriae of *B. fragilis* are still in their infancy. This may be due to technical difficulties in observing fimbriae by electron microscopy (EM) or a lack of understanding of the precise growth conditions required to favour assembly of fimbriae. The slow progress in characterization of the fimbriae expressed by *B. fragilis* may be a clue to the possibility that structural variation of fimbriae also occurs.

The first report of fimbriae in the published literature was as recent as 1984 when Pruzzo and colleagues described filamentous appendages of approximately 30nm in diameter.[34] Subsequently, van Doorn and colleagues described fimbriae of 4–5nm in diameter.[35] These authors raised polyclonal antiserum to the fimbriae with which they succeeded in immuno-gold labelling the fimbriae. Immunoblotting revealed a fimbrial subunit of 40–42 kDa, depending on the strain. They showed that fimbrial expression was reduced at low iron concentrations and low temperature.

We have been able to detect the fimbrial subunit by immunoblotting of bacteria grown in vivo and in the LC, SC and EDL populations in various strains;[36] however, we have only observed intact fimbriae by EM and negative stain after the bacteria have been cultured on agar plates, and not after broth culture. It seems likely that other workers have experienced difficulty in observing fimbriae on *B. fragilis*. Possible explanations for the problems in studying fimbrial expression include the interference of polysaccharide in detection of the fimbriae. Long strands of material are observed when *B. fragilis* is platinum-gold shadowed[36] or coated (Fig. 5) after broth culture. The question remains as to whether these are strands of polysaccharide or polysaccharide covering underlying protein structures. Polysaccharide could also mask the epitopes on the fimbriae, making immunogold labelling erratic and difficult. It may be that fimbrial subunits are expressed, but not always assembled into intact fimbriae. This underlines the need for extensive genetic studies of the expression and control of expression of fimbriae in *B. fragilis*. Van Doorn and colleagues have started on this road by determining the N-terminal sequence of their 40–42 kDa fimbrial subunit. Interestingly it does not belong to the N-methyl phenylalanine family of fimbriae characteristic of bacteria such as *B. nodosus, Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* (van Doorn et al, personal communication).

Virulence: mechanisms

Immunomodulation by lipopolysaccharide

The lipopolysaccharide of *B. fragilis* characteristically responds poorly in the tests usually performed to examine the activity of LPS from enteric bacteria such as *Salmonella* spp and *E. coli*. It is about 10- to 10000-fold less active in limulus lysate gelation, pyrogenicity, lethality to mice, toxicity to chicken embryo and the dermal Shwartzman reaction.[37] In spite of this, aqueous phenol extracts of *B. fragilis*

polysaccharides are not totally without immunomodulatory activity. Extracted polysaccharides were mitogenic for spleen cells derived from mice strains which respond to enterobacterial endotoxin.

Whether strains of LPS-hyporesponsive mice, which carry a mutation on the 4th chromosome in the *Lps* gene and do not respond to lipid A from enterobacteria, respond to *B. fragilis* LPS, remains an open question. Extracted *B. fragilis* polysaccharide is mitogenic for spleen cells from these mice at high spleen cell density;[37,38] however, other immune reactions of these hyporesponsive mice remain low.[38] Unfortunately the extraction procedures used in these studies would have produced a mixture of polysaccharides, rather than purified LPS.

For many years *B. fragilis* LPS was thought to lack the sugar keto-deoxyoctonate (KDO) in the core sugar region, as were the oral bacteroides such as *Porphyromonas (Bacteroides) gingivalis*. This assumption was due to a lack of reactivity of the LPS in the thiobarbituric acid (TBA) method used to detect KDO. This lack of reactivity stems from phosphorylation of the KDO.[33,39] Dephosphorylation of the LPS results in reactivity in the TBA assay. This unusual KDO, the lack of phosphorylation of the glucosamine disaccharide backbone of the lipid A and differences in the fatty acid composition may all contribute to the lower toxicity of bacteroides LPS.

The role of components of *Bacteroides* spp in endotoxic shock, however, remains to be defined. Although the LPS has a lower activity than enterobacterial endotoxin, *Bacteroides* spp are numerically predominant in faeces and may therefore be as important in endotoxic shock as are the enterobacteria. Perhaps the role played by *Bacteroides* spp may become more evident as treatments based on MAbs to *E. coli* LPS come into more general clinical use.

Attachment to host cells

Numerous studies of the attachment of *B. fragilis* to host cells, such as tissue culture cell lines and erythrocytes, have yielded apparently conflicting results. Both polysaccharides and fimbriae have been reported to be involved and not involved in attachment to host cells (see [35,40–42]). The results become less confusing if it is accepted that *B. fragilis* can produce more than one type of ligand to mediate host cell attachment and that expression of these ligands is subject to within-strain variation. In our studies, we have shown that by mixing together different proportions of bacteria with either the LC or EDL it is possible to correlate the degree of haemagglutination with the proportion of EDL bacteria within a given population.[43] We have also shown that released extracellular vesicles (ECV) will cause haemagglutination (unpublished result). It is therefore essential to be aware of, not only the surface structures expressed by the populations being examined, but also the amounts of ECV and extracellular polysaccharide (EPS) present. If quantities of ECV or EPS are present they may block attachment sites on tissue culture cell lines and therefore exclude the bacteria. In assays which rely on quantifying the attached bacteria, erroneous negative results may be obtained. Despite the confusion in identifying which particular structures are involved in attachment to host cells, it can be concluded that *B. fragilis* can successfully attach to host cells. This ability would be clearly advantageous in situations where the host's secretions will wash the bacteria away.

Recent studies have shown that under certain conditions *B. fragilis* may penetrate host cells.[44] This may be particularly important in bacteraemia, where the route of travel of the *B. fragilis* from an initial focus of infection in, for example the peritoneal cavity, to the bloodstream is unclear.

Nutrition in vivo: release of extracellular degradative enzymes

B. fragilis have been reported to release extracellular enzymes capable of degrading the components of the host's extracellular matrix (e.g. hyaluronidase, chondroitin sulphatase), host cells and tissues (e.g. DNAase, lipase, protease and neuraminidase).[45] Constitutive expression of a range of periplasm or outer membrane associated proteases with arylamidase activity towards the amino acids leucine, valyl and glycyl-proline has also been reported.[46]

In spite of this, *B. fragilis* is not a progressive, fast-spreading destructor of the host as is, for example, *Clostridium perfringens*. It seems likely that the enzymes released by *B. fragilis* act in a localized area, although some enzymic activity has been observed in association with released ECV (unpublished result).

Neuraminidases, which cleave sialic acid from oligosaccharides on host cell glycoproteins and glycolipids, may have a more subtle effect on the host. There are more than 20 known naturally occurring sialic acids formed by various substitutions and additions to neuraminic acid.[47] There is growing evidence that these sugar residues are involved in the biological activities of the host cells and molecules.[48] A prime example is the movement of lymphocytes within the host.[49] Therefore the released neuraminidase enzymes may subvert the normal function of the immune system or other systems of the host. The *nanH* which encodes for neuraminidase in *B. fragilis* has been successfully cloned in *E. coli*.[50] Perhaps studies of the virulence of *B. fragilis* lacking this gene will shed more light on the role of neuraminidase in pathogenesis.

Nutrition: iron uptake mechanisms

For *B. fragilis* to grow in vivo, it must be assumed that it is able to scavenge iron successfully from the proteins and glycoproteins to which iron is normally

complexed in the host. The growth of *B. fragilis* in complex tryptone yeast extract medium in the presence and absence of the iron chelating agent 2,2' dipyridil was compared by Otto and colleagues. Novel outer membrane proteins (OMP) of M_r 89, 49, 44 and 23.5 kDa were detected only under the iron-limited conditions.[51] Studies with a defined medium, which was depleted of iron by precipitation with $CaCl_2$, showed that *B. fragilis* grown in this medium had a requirement for protoporphyrin. This was related to the presence of the 44 kDa OMP. Further experiments indicated that haemoglobin was the best utilised source of iron when apotransferrin was present in the iron-depleted medium. It therefore appears that in vivo *B. fragilis* may use haem from haemoglobin as an iron and protoporphyrin source.[52] Interestingly, in the defined medium used, the maximum optical density in late exponential phase cultures, even under iron and protoporphyrin replete conditions, was approximately 1.5 at OD_{650}. This strongly suggests that something else within the growth medium is also limiting growth.

Studies of both normal human sera, and sera from rats which had been experimentally infected with *B. fragilis* indicated that there was a specific antibody response to a 44 kDa OMP. This implies that this protein is indeed expressed in vivo.[53] At present there is no evidence that *B. fragilis* produces its own chelating agents. The possibility of having uptake mechanisms for the chelating agents of, for example, *E. coli* has not been ruled out. A comparison of *B. fragilis* growing in vivo and in vitro, however, did not reveal the induction of copious quantities of high M_r OMP receptors for chelating agents which are characteristically produced by *E. coli* under iron limitation.[54]

These studies highlight a greater need for attention to the basic physiology of *B. fragilis* when grown under different nutritional conditions and a need to compare in vivo and in vitro grown cultures.

Evasion of phagocytosis and complement activity

The large capsule of *B. fragilis* impairs phagocytic uptake and killing by human polymorphonuclear leukocytes in vitro, probably by mopping up host opsonins. Although the EDL population is readily phagocytosed in vitro, this population is resistant to killing by normal human serum.[54] Unexpectedly, the large capsule is selected against in a mouse model of infection, in the presence of phagocytic cells;[22] however, the population which is selected excretes copious amounts of extracellular polysaccharide, which shares epitopes with the LC.[20] It seems likely that if sufficient EPS is excreted in vivo it may also mop up opsonin, thereby reducing phagocytic activity. The release of ECV may have a similar function, causing the deposition of opsonins and perhaps activation of complement at a distance from the bacterial cell surface. ECV are produced in a natural infection as they can be detected after immuno-labelling by both fluorescence and electron microscopy directly in pus samples (unpublished result). Pathogenic synergy, whereby *B. fragilis* protects and enhances the survival of other facultatively anaerobic bacteria in an infection, is known to occur. The depletion of opsonin[56] and release of factor(s) which inhibit the chemotaxis of polymorphonuclear leukocytes, such succinic acid,[57] are implicated. The EPS may also coat other facultative bacteria within a polymicrobial infection and contribute to the synergy. Interestingly, abscess-derived neutrophils are reported to harbour viable bacteria and be less efficient at killing bacteria than neutrophils derived from either peritoneal aspirates or peripheral blood, in in vitro experiments.[58]

In a comprehensive study of the opsonization of *B. fragilis*, using isolated components of the alternative complement pathway (ACP), Bjornson et al[59] demonstrated that C3 deposition occurred with C3, properdin, factors H, I, B and D alone. Attachment to polymorphononuclear leukocytes, uptake and killing, however, required other as yet unidentified 'auxilliary' factors present in serum. These results indicate that resistance of *B. fragilis* to either complement-mediated lysis or phagocytic uptake and killing is not mediated by a lack of activation of the ACP or C3 deposition. Unfortunately the surface structures of the bacterial populations used in this study were not defined.

Antigenic variation of surface carbohydrates could play a significant role in the evasion of the specific immune response. Bacteria taken directly from pus samples[60] and from an animal model of infection[21,22] are antigenically heterogeneous when labelled with carbohydrate specific antibodies.

Conclusion

Despite strenuous effort by many workers, the precise virulence determinants of *B. fragilis* remain elusive. Part of the reason for this is that within-strain variation of both surface structures and antigenicity has been overlooked. Thus, the use of heterogeneous populations has led to apparently conflicting results.

Surface variation may well turn out to be as important in the virulence of *B. fragilis* as it is in other well-studied pathogenic bacteria.

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REVIEWS IN MEDICAL MICROBIOLOGY

Haemophilus species and clones

T.H. Pennington

Department of Medical Microbiology, University of Aberdeen, Medical School, Aberdeen AB9 2ZD, UK

Summary. The current taxonomic position of *Haemophilus* species that infect man is reviewed, with particular reference to *H. influenzae* and *H. parainfluenzae*. Recent studies on the population genetics of *H. influenzae* are reviewed. Evidence that the population structure of this species is clonal is appraised in detail, and a general account is given showing how the techniques of population genetics and molecular systematics are used for the clonal analysis of bacterial populations.

Introduction

Most medical microbiologists consider that they have a clear understanding of attributes, boundaries, members and biology of the genus *Haemophilus*. According to Bergey's Manual[1], it is defined as comprising Gram-negative rods or coccobacilli, which require preformed growth factors present in blood, namely X factor (protoporphyrin IX or protoheme) and/or V factor (nicotinamide adenine dinucleotide (NAD) or NAD phosphate). Bergey lists 10 species from man, two from pigs, two from poultry and one from the dog, all of which are obligate parasites. Of the species that colonize man, *H. influenzae* is by far the most important clinically, in that it causes a number of severe and quantitatively important infections. *H. influenzae* is also distinctive in that it can undergo genetic transformation with high efficiency.[2]

The purpose of this review is to consider these statements in the light of recent work on the population genetics of *Haemophilus* species and the clonal concept of bacterial population structure.

A number of reasons make *Haemophilus* a particularly appropriate and interesting subject for discussion under these headings. Noteworthy, for example, is the extreme importance played by the requirement for X and V factors in the definition and subdivisions of the genus which in taxonomic terms makes it virtually monothetic, a characteristic considered undesirable by systematists[3]. In addition, recent work has shown that natural populations of *H. influenzae* are probably more variable genetically than those of any other prokaryote or eukaryote species, thus making the organism as distinctive as the cheetah, which shows no genetic variability at all.[4] Their positions at the extremes of variation make both species particularly interesting to anyone who wishes to investigate their evolution, speculate about their natural history, and make predictions about how their populations will change in the future. The work on the population genetics of *H. influenzae* has also revealed that its populations, like those of many other important bacterial pathogens, have a structure which is basically clonal.

Haemophilus species

The genera *Haemophilus, Pasteurella* and *Actinobacillus* together form the family Pasteurellaceae[5]. Identification of the genus *Haemophilus* by determining a requirement for X factor or V factor or both, whilst very useful as an operational characteristic in the diagnostic laboratory, is unreliable for all strains. It is therefore unacceptable as an exclusive definitive feature. Consideration of the porcine organism *Haemophilus pleuropneumoniae* provides an illustrative example of the taxonomic difficulties that have arisen in this area. This organism generally has a growth requirement for V factor. However, phenotypically it has been shown to resemble a *Pasteurella haemolytica*-like organism which causes porcine necrotizing pleuropneumonia, and DNA hybridization of the

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6: Attachment in Disease

S. PATRICK[1] AND M.J. LARKIN[2]
[1]*Department of Microbiology and Immunobiology, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN; and* [2]*School of Biology and Biochemistry, The Queen's University of Belfast, Belfast BT9 5AG, UK*

The attachment of pathogenic bacteria to mammalian cells is a pivotal step in the colonization of the host. Attachment is mediated by the interaction of ligands on the bacterial surface and receptor molecules on the mammalian cell. Bacteria have evolved with a range of attachment mechanisms, from the non-specific in terms of cell type and cell surface receptor, to the highly specific. Virtually all of the known structures on the surface of bacteria are involved in some way in attachment to target cells, either by promoting or interfering with attachment. For example, bacterial surface structures may impede attachment and uptake by phagocytic cells. On the other hand, attachment may be a prelude to the phagocytosis of the bacterium by a normally non-phagocytic host cell.

The structures involved are fimbriae (sometimes referred to as pili), flagella, lipopolysaccharide (LPS), extracellular polysaccharides in the form of capsules or glycocalyx, lipoteichoic acids and outer membrane proteins (OMP). Fimbriae, first named by Duguid *et al.* (1955) after the Latin for 'thread' or 'fibre', are generally accepted as very important in bacterial attachment to host cells. Brinton (1959) named similar structures pili after the Latin for 'hair-like' and both names are still in use. Expression of such adhesive structures, or adhesins, by the bacteria may be regulated by environmental changes associated with the host cells or the host cells themselves. In the case of *Salmonella* spp., an initial attachment event triggers the biosynthesis of further adhesin molecules (Finlay *et al.*, 1989).

The interaction of the bacterial adhesin with the host cell can also alter the functions of the host cell. These may include the induction of plasmin formation, rearrangement of the cytoskeleton to induce phagocytosis of the

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bacterium and, possibly, cytokine release (Hoepelman & Tuomanen, 1992).

Studies of attachment in disease must consider the characterization of the relevant surface structures on the bacteria, the interaction between the bacterium and the target cell surface and the characteristics of the target cell surface. Such studies necessarily involve a wide range of biochemical and microbiological techniques too numerous to detail here, and the reader is directed to Hancock & Poxton (1988) for a full account. This chapter will deal with techniques and approaches adopted in our work on the virulence of the obligate anaerobe *Bacteroides fragilis*, in relation to the identification of surface structures by microscopy and their antigenicity. This will provide an insight into the likely problems and factors to be considered when approaching the study of a pathogen for the first time.

Characterization of Surface Structures by Microscopy

Before embarking on any study of bacterial attachment it is essential to define the surface structures expressed on the surface of the organism, and to ensure that the population to be studied is homogeneous with respect to expression of the surface structure of interest. The antigenic heterogeneity of the structures should also be examined because the parts of the bacterial molecule involved in attachment may not be expressed by all of the bacteria within a population. For example, Type 1 fimbriae, involved in attachment by *Escherichia coli*, may be subject to both antigenic and phase variation. In the latter there is a switch from expression to non-expression of the fimbriae at a rate of 10^{-2}–10^{-3}/cell/generation as the result of inversion of a 314 base-pair segment of DNA (Tennent *et al.*, 1990). Considerable heterogeneity has been shown with respect to surface structure/antigen expression in *B. fragilis* (Patrick & Lutton, 1990). Subpopulations of bacteria, separated by density gradient centrifugation from a single strain, have different haemagglutinating properties, which suggests that attachment studies should be approached with caution (Patrick *et al.*, 1988). A number of simple precautions should, therefore, be taken. First, the bacteria should be grown in a defined medium to facilitate the control of available nutrients. For example, nutritional conditions can alter the size of capsules expressed by *E. coli* (Sutherland, 1977). Secondly, the phase of growth of the culture should be known. Stationary-phase cultures should be avoided because such populations are not homogeneous for any metabolic parameter, and some of the population will be either dead, dying or utilizing nutrients released from dead cells. Ideally, bacteria in continuous culture should be used to ensure their metabolic uniformity. Alternatively, cultures should be inoculated from a standard inoculum, stored in batches at −70°C or in liquid nitrogen, and always harvested at a particular phase of growth,

such as the late exponential phase. Thirdly, variation in the expression of surface structures with growth phase should also be determined. Finally, bacteria grown *in vivo*, either in an experimental model (e.g. Day *et al.*, 1980; Patrick *et al.*, 1984), or taken directly from clinical specimens, must be examined for the expression of surface structures implicated in attachment by the *in vitro* studies.

The following methods and techniques are regarded as generally appropriate for any bacterial population to be studied.

Light microscopy

Negative staining with India ink in a wet mount and observation by light microscopy should be undertaken to determine whether capsules are present on the bacterium of interest. This is an essential first step which lacks the drawback of dry preparations, where shrinkage of both the negative stain and the bacterial cell may give false-positive results. Once the presence of capsules has been established, dry staining methods can then be compared. If the latter give the same results, they can be used in further studies. Dry staining methods are more useful if time is limited and a number of samples are to be examined, because prepared slides can be stored in the dark at room temperature and examined later.

Wet India ink method (Cruickshank, 1965)

A large loopful of India ink is mixed on a clean glass slide with a small quantity of broth culture or bacteria taken from a colony. A coverslip is then placed on the suspension and pressed down hard between sheets of blotting paper. The preparation is viewed by phase-contrast microscopy at $\times 1000$ magnification. Encapsulated bacterial cells should be seen surrounded by clear areas corresponding to the capsule, with a background of India ink particles.

Dry eosin/carbol fuchsin method (Cruickshank, 1965)

Carbol fuchsin staining solution (Ziehl–Nielsen's carbol fuchsin diluted 1:5) has the following composition: basic fuchsin (5 g), absolute ethanol (40 ml), phenol (5% w/v in distilled water; 500 ml). The fuchsin is dissolved in the alcohol and is added to the phenol solution. For capsule staining this solution is further diluted 1:5 in distilled water and can be stored in the dark at room temperature. Eosin staining solution consists of four parts eosin solution (10% w/v water-soluble yellowish or bluish erythrosin in distilled water) and one part serum (human, rabbit, sheep or calf) heated at 56°C for 30 min. A crystal of thymol may be added as a preservative. After standing at room

temperature for a few days, the mixture is centrifuged and the supernatant is retained. It is stable at room temperature for at least a year.

Staining method. To stain the bacterial cells mix one drop of broth culture or bacterial suspension in one drop of carbol fuchsin on a clean glass slide and allow to stand for 30 s. Then add one drop of eosin solution and leave for 1 min. Spread a film of the suspension along the slide with a second clean slide, as in preparing a blood smear, allow to dry at room temperature, and examine by light microscopy at ×1000 magnification. The bacteria, particularly those without capsules, will be much clearer if bright-field phase-contrast microscopy is used. This gives the bacteria a greenish tinge against the pink background of the eosin. The matt background of the eosin stain can be enhanced by adding a loopful of Percoll (20% in saline; Pharmacia Biosystems (UK) Ltd, Milton Keynes, UK) to the bacterial suspension on the slide. Figures 6.1(a, b and c) illustrate bacteria stained using the eosin carbol fuchsin technique.

Loops should be carefully flamed and allowed to cool before dipping into the staining solution. For both staining techniques control slides should be periodically prepared without bacterial culture, to ensure that the stains have not become contaminated.

Electron microscopy

Electron microscopy is an essential tool which will reveal capsules not visible by light microscopy, and also whether fimbriae are present on the bacterial surface.

Ultrathin sectioning of embedded bacteria

This method can be used for determining the presence of capsular material, and a variety of different methods for embedding, fixation and staining is available. The method chosen is usually determined by trial and error, because different types of bacterial polysaccharide capsule may have different properties. Ruthenium red staining after osmium tetroxide and glutaraldehyde fixation is useful because ruthenium red stains negatively charged polysaccharides. For successful staining, commercial ruthenium red should first be purified to remove contaminating ruthenium purple. The use of specific antibody to stabilize capsules has the disadvantages that structural detail of the capsular material is lost, and it precludes subsequent immunolabelling to identify particular structures. If immunolabelling of ultrathin sections is to be carried out, osmium tetroxide fixation and ruthenium red stain may destroy the antigenicity of the polysaccharide epitopes. For this reason glutaraldehyde/

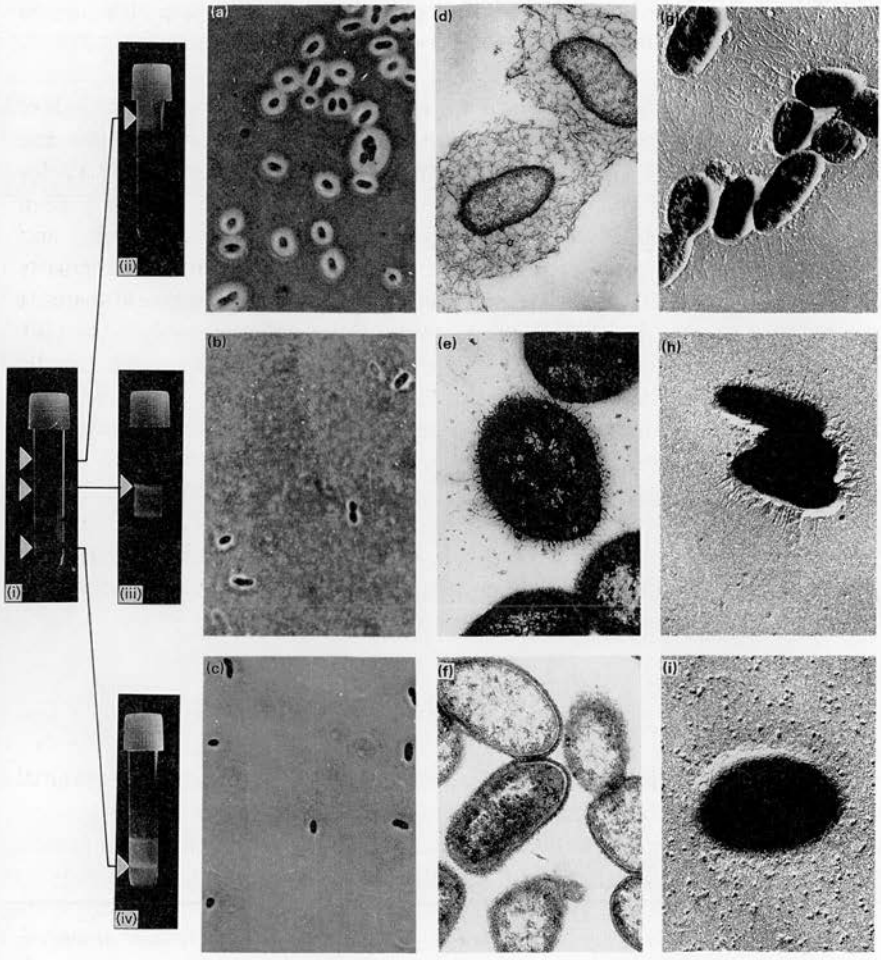

FIG. 6.1. Appearance of *Bacteroides fragilis* enriched by Percoll density gradient centrifugation. (i) Unenriched broth; after overnight subculture (ii) from 0–20% interface layer (large capsule population); (iii) 20–40% interface layer (small capsule population); (iv) 60–80% interface layer (electron-dense population). Micrographs of large capsule population cells (a, d, g); small capsule population cells (b, e, h); non-capsulate (electron-dense layer) population cells (c, f, i). Eosin/carbol fuchsin-negative stain (a, b, c); ultrathin section electron microscopy (d, e, f); platinum–gold shadowing electron microscopy (g, h, i).

paraformaldehyde fixation is used, but this has the disadvantage that capsules are no longer visible. If facilities are available for cryofixation and freeze substitution embedding, the appearance of capsular material may be retained and immunogold labelling may still be successful. The use of Lowicryl K4M resin and dimethyl formamide as a dehydrating agent may also allow visualization of capsular material with concomitant immunogold labelling (Bayer, 1990).

Purification of ruthenium red (after Luft, 1971)

1 Place 0.25 g of commercially available ruthenium red in a mortar with a few drops of 0.5 mol/l ammonia solution and grind with a pestle. Transfer the suspension to a V-shaped quick-fit test tube. Adjust the final volume to 10 ml.
2 Incubate the solution at 60°C for 30 min in a water bath, with frequent vigorous stirring with a glass Pasteur pipette.
3 Cool the test tube under tapwater and centrifuge in a bench centrifuge at 1500 *g* for 5 min.
4 Carefully withdraw the supernatant fluid and place in a clean 50-ml beaker.
5 Allow the supernatant to evaporate in a desiccator containing 100 g each of anhydrous $CaSO_4$, NH_4CO_3 and NaOH.
6 Store in the desiccator in the dark until ready to use.

Fixation and staining procedure

1 Wash bacteria in cacodylate buffer (0.1 mol/l, pH 6.8) or add a small quantity of bacteria directly to a large volume of fixative/stain.
2 Incubate in fixative/stain of glutaraldehyde (2.5% v/v), ruthenium red (1 mg/ml) in cacodylate buffer (0.1 mol/l) for 1 h at 4°C in the dark.
3 Wash three times in buffer. The bacterial suspension will form a granular pellet. Do not break this up but try to keep it as granular lumps throughout the further procedures by stirring gently with the end of a Pasteur pipette, rather than by sucking up and down. This greatly reduces the loss of material during the procedure.
4 Resuspend in osmium tetroxide (1% w/v), ruthenium red (1 mg/ml) in cacodylate buffer, as before. Incubate for 3 h at room temperature in the dark. Wash twice in buffer.
5 Dehydrate in graded ethanol by centrifuging and suspending sequentially in 30, 50, 75, 95 and 100% ethanol. Repeat the 100% ethanol treatment. Filter the 100% ethanol through sodium sulphate on Whatman No. 1 filter paper immediately before use to ensure that it is completely dry. Ensure that the samples are not in any alcohol solution for longer than 15 min, including the centrifugation period, though this limits the number of samples that can be processed at any time.
6 Infiltrate and embed in the resin of choice — LR White is one of the

simplest to use and is compatible with immunogold labelling of ultrathin sections. After the final dehydration step, the pelleted bacteria are suspended in resin–dried 100% ethanol (1:1) for 1 h and then left overnight in 100% resin. The resin should be changed two or three times during the next day. The samples should always be in containers left open to the air as the resin, if enclosed, may begin to polymerize prematurely. Finally, place the sample, in resin, into a gelatine or Beem capsule (Agar Scientific Ltd, Cambridge, UK) and gently centrifuge to pellet the sample. A paper label can be inserted into the resin before polymerization by incubation at 60°C overnight.

7 Ultrathin sections can then be cut, stained — for example with uranyl acetate — and viewed by electron microscopy.

Negative staining of whole cells for electron microscopy

This is useful to determine the presence of fimbrial structures and flagella. Glow-discharging of formavar/carbon-coated grids, within about 30 min of use, will improve the retention of material on the grid. Grids are dipped into the bacterial suspension in distilled water, allowed to drain on filter paper and then dipped into a drop of stain, such as methylamine tungstate 2% (w/v) in distilled water, and again allowed to drain. Immunogold labelling may be carried out before negative staining. Negative stain may also reveal condensed capsular material as a result of the dehydration of the polysaccharides, which generally contain 99% water. The interpretation of the appearance of this condensed material is frequently difficult. The drying that occurs as a result of placing the bacterium on the grid and into the microscope vacuum, as opposed to the controlled dehydration after fixation and staining for thin section, may not always give this material a consistent appearance.

Platinum–gold shadowing of whole cells

This is similar to negative staining in that whole cells are placed on an electron microscopy grid and shadowed at an angle (e.g. 20°) with platinum and gold in a high-vacuum coating unit such as a Balzer BAE 120 (Lutton *et al.*, 1989). Again, interpretation of the structures observed may be difficult because dried capsular material may have a fibrillar appearance (see Figs. 6.1(g) and (h)). Definition of the structures associated with the surface of the bacterium should ideally involve an electron microscopic comparison of whole cells and thin sections, as well as light microscopy. Figure 6.1 compares three populations as seen by light microscopy ((a), (b) and (c)), electron microscopy of ultrathin sections ((d), (e) and (f)) and platinum–gold shadowed whole bacteria ((g), (h) and (i)). The main points to note are that (i) the large and small capsules after platinum–gold shadowing are fibrillar in appearance; (ii) the bacteria, which

are apparently non-capsulate by light microscopy, have a marginal electron-dense layer in ultrathin sections; this layer is termed electron-dense because it is visible by electron microscopy in both the presence and absence of ruthenium red, and suggests that it may have non-polysaccharide components (Patrick *et al.*, 1986); and (iii) the small round objects visible after platinum–gold shadowing in both the electron-dense layer population and within the 'fibrils' of the large capsule are extracellular vesicles that bud from the outer membrane (Lutton *et al.*, 1991). If these are present in a bacterial population, their possible involvement in or interference with attachment of the bacteria to host cells should be taken into account.

Immunological Characterization of the Surface Structures

Definition of the antigenicity of the observed surface structures facilitates the study of the antigenic variation that may be evident within strains. Ideally, monoclonal antibodies (MAbs) specific for particular surface structures should be prepared. An excellent account of the production of MAbs is given by Goding (1986). Immunofluorescence microscopy and flow cytometry can then be used to monitor antigenic variation. Immunogold electron microscopy will assist in locating the epitope within the bacterium. Methods for immunogold labelling are provided by Palak & Varndell (1984) and Beesley (1989).

Immunofluorescence microscopy

Preparation and coating of slides

Multiwell slides (Flow Laboratories Ltd, Rickmansworth, UK) are soaked overnight in a weak solution of Decon (approx. 0.1% v/v), thoroughly rinsed in tapwater and then in distilled water, and dried. A slightly turbid suspension (30 µl) of bacteria diluted in phosphate-buffered saline (PBS; (g/l) NaCl, 8.00; K_2HPO_4, 1.21; KH_2PO_4, 0.34; pH 7.3) is placed in each well. Optimum concentrations of bacteria should be determined empirically, so that an even distribution of a single layer of bacteria is seen on microscopy. Slides are dried at 37°C in a fan incubator and fixed by placing them in 100% methanol at −20°C for 10 min. The slides are then dried at room temperature and wrapped with a sachet of silica gel. They may be stored at −70°C for many months, depending on the stability of the antigens.

Two-step immunolabelling procedure

This is based on Johnson *et al.* (1978). On removal from storage at −70°C, the slide is allowed to come to room temperature. Primary antibody (30 µl),

diluted in PBS or neat hybridoma supernatant, is applied to the well. The slide is then incubated at 37°C in a humidified plastic box. From this stage onwards the wells must *not* be allowed to dry out.

The primary antibody is then washed off with PBS with a wash bottle. Care must be taken to ensure that antibody from one well does not run over into another. The PBS is therefore directed to the centre of the slide, so that antibody is washed over the outer edge of the slide.

The slide is then placed in a bath (e.g. a glass staining jar) of PBS and mixed with a magnetic stirrer for at least 30 min. The back of the slide should face the magnetic 'flea', in case it damages the bacterial film. The back of the slide is then dried. The spaces between the wells are also gently dried with a strip of filter paper; the wells themselves must not be dried.

The appropriate dilution of secondary antibody (e.g. antimouse IgG fluorescein isothiocyanate (FITC) conjugate; 30 μl) is then applied to the well and the incubation and washing steps are repeated. Finally, the slide is covered with a mounting fluid, such as 10% (w/v) glycerol in PBS, containing an antibleaching agent (e.g. photobleaching retardant; Syva Corporation, USA) and covered with a large coverslip. If this is sealed with nailvarnish, the slides can be stored for a few weeks at 4°C in the dark.

Dual labelling can be carried out by adding an extra step. After applying the primary antibody, such as a murine MAb, and washing, a rabbit polyclonal antiserum, known to label all the bacteria within a population, may be added. After washing, the bacteria are incubated with two conjugates together, such as FITC-conjugated antirabbit immunoglobulin and tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated antimouse immunoglobulin. The slide is then mounted as above. The bacteria can be examined by fluorescence microscopy with the appropriate filters. The double label allows the detection of non-homogeneous labelling of a bacterial population by a MAb. Double exposure of colour film with the same field of view and with filters suitable for FITC and TRITC will give pictures of a single double-labelled field. A simpler method is to observe the same field by phase-contrast microscopy and fluorescence microscopy.

Preparation of Homogeneous Populations

Once structural or antigenic heterogeneity has been established for a bacterial species, consideration must be given to methods for producing homogeneous populations, before attachment is studied. Two methods are outlined below.

Physical size and density gradient centrifugation

Density gradient centrifugation can be used to separate bacteria that express

different surface structures. Discontinuous density gradients, also referred to as step gradients, can be prepared with sucrose or other materials, such as Percoll (Pharmacia Biosystems (UK) Ltd, Milton Keynes, UK). The latter has the advantage that it is supplied sterile and, when diluted, can be made isotonic with physiological saline at a physiological pH. The bacterial populations can be separated on a bench centrifuge, rather than by ultracentrifugation. In this way, bacteria suspended in Percoll retain their viability. Bacteria harvested from the gradients can be used either directly or, if expression of the surface structures is relatively stable, they can be subcultured before assays of attachment.

Isopycnic density centrifugation has been used to separate *E. coli* possessing colonization factor I (Giesa *et al.*, 1982). Continuous gradients were prepared in an ultracentrifuge, and the bacterial populations were then applied to the gradients and centrifuged on a bench centrifuge. Discontinuous gradients may be prepared as detailed below, and have been used to separate *B. fragilis* with different sizes of capsules (Patrick & Reid, 1983; it should be noted that photomicrographs b and c in this publication are transposed). Since each bacterium may vary, empirical studies should be carried out with different percentage dilutions of Percoll and different centrifugation times. A list of publications in which Percoll has been used may be obtained from Pharmacia at the above address.

Preparation of Percoll

If bacteria are to be cultured after separation, the following manipulations must be conducted under aseptic conditions. Percoll is supplied sterile and should be dispensed aseptically in 90-ml volumes and stored at 4°C in sterile bottles sealed with tape.

Percoll is made up in 0.15 mol/l sodium chloride and this will be called '100% Percoll'. A small volume is removed and the amount of 1 mol/l HCl required to bring the pH to approximately 7, based on the number of drops from a standard 20 µl dropper, is noted. The Percoll should be well mixed as each drop of HCl is added, and will become more cloudy in appearance. The required volume of HCl per millilitre of the 100% Percoll is calculated, sterile HCl is added aseptically, a small volume is removed and the pH determined. The required volume of HCl per 100 ml Percoll should remain constant within a single batch of Percoll, but this may vary between batches and should always be rechecked.

Preparation of gradients

The 100% Percoll (pH 7) can be further diluted with 0.15 mol/l NaCl to give 80%, 60%, 40% and 20% (v/v) suspensions. These are then placed into

11 ml/16 mm diameter sterile plastic screw-capped tubes, starting with 80% at the bottom. The other layers, in decreasing concentration, are then added, 2 ml per layer. If only a few tubes are required the gradient steps can be layered carefully by hand with a Pasteur pipette. The initial contact of one layer with another is critical for the integrity of the interface. If the Percoll is added too quickly the layers will mix. The Percoll should be allowed to run gently down the side of the tube on to the surface of the layer below. If large numbers of gradients are required, a peristaltic pump can be used to prepare them. Stiff narrow-gauge tubing should be inserted into the soft tubing passing through the pump. To ensure asepsis, 70% (v/v) industrial methylated spirits in water should be passed through the tubing, followed by sterile saline. After preparation the interfaces should be clearly visible. Less mixing between layers occurs if the Percoll suspensions are used straight from the 4°C refrigerator. If the refrigerator is vibration-free, gradients can be stored overnight at 4°C. The interfaces will be less obvious on the second day, but the system will work adequately.

Separation of bacteria

With a Pasteur pipette *ca.* 2–2.5 ml of a broth culture is carefully layered on to the gradient, which is centrifuged in a bench centrifuge with a swing-out head. The relative centrifugal force and the time of centrifugation for a particular bacterium will vary, and can only be determined empirically: between 2000 and 3000 *g* for 20–40 min is a good starting point. First, after centrifugation, the culture medium on top of the 20% layer is removed with a clean pipette and bacteria are then removed from each of the interfaces with a Pasteur pipette. Percoll in the 20% layer is then carefully removed, without disturbing the 20–40% interface, and discarded. Bacteria at the 20–40% layer are removed next with a clean pipette, the 40% Percoll is discarded, and so on down the gradient. The use of a clean pipette and discarding of the Percoll between the interfaces minimizes the contamination of one layer with another. Even if a distinct band of bacterial culture is not visible at an interface, it should still be removed, examined by microscopy and subcultured. In the case of *B. fragilis* there may be too few cells with a large capsule to be visible as a band on top of the 20% Percoll. They can, however, be enriched by subculture from the interface (Fig. 6.1(i) to (iv)). Since the enrichment may not be absolute, it is wise to repeat the density gradient centrifugation after culture from the interface layers.

Immunological separation

The use of immunomagnetic beads is a simple method for enriching bacteria that express particular surface epitopes. Immunomagnetic beads can be bought

already conjugated to either antimouse or antirabbit immunoglobulin. If MAbs are available for particular epitopes, these can be attached to the immunomagnetic beads and then used to remove bacteria carrying that epitope from a mixed population (Fig. 6.2). Since it is difficult to remove the bacteria from the beads, this technique relies on the subculture of the bacteria and enrichment of bacteria expressing the epitope.

Coating of antimouse IgG-magnetic beads with murine monoclonal antibody

The precise volumes of antibody to be used will depend on its titre. We have used the following procedure with neat supernatant from a hybridoma cell line. Immunomagnetic beads (50 µl; Dynabead M-450, coated with antimouse IgG; Dynal Ltd, New Ferry, Wirral, UK) are pipetted into a 1-ml glass test tube. The tube is clipped to a plastic-coated magnet called a magnetic particle concentrator (MPC; Dynal Ltd) for at least 1 min. While the test tube is still clipped to the MPC, the fluid is removed with a pipette and 5 ml sterile PBS

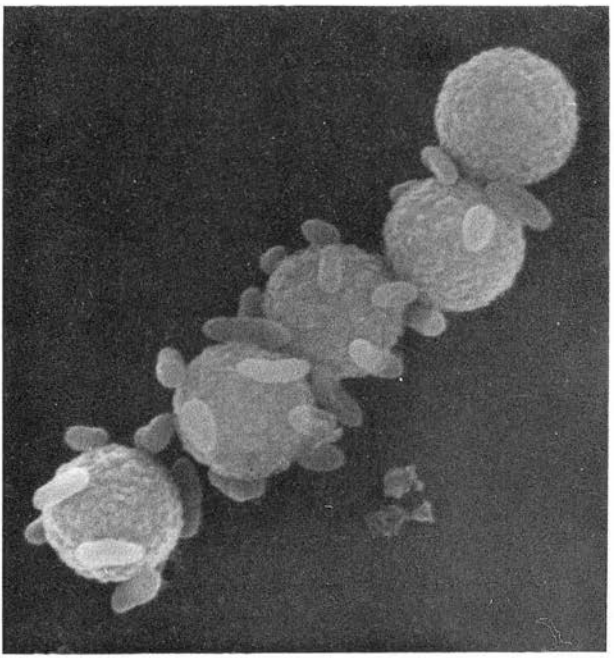

FIG. 6.2. *Bacteroides fragilis* attached to antimouse IgG-coated immunomagnetic beads via an anti-*B. fragilis* polysaccharide mouse monoclonal antibody.

is added. The test tube is removed from the influence of the magnet and the contents are vortexed. The tube is then replaced in the MPC and the PBS removed and replaced. This process is repeated twice. These washing steps remove the sodium azide in which the beads are supplied. The beads are finally made up to 50 µl in PBS and again vortexed. Monoclonal antibody supernatant (1 ml) is added and the beads are incubated at room temperature for 2–24 h with very gentle rocking. The beads are then washed again four times in PBS as above, with gentle rocking for 30 min each time. They are finally suspended in 1 ml PBS, and can be stored at 4°C for up to 2 weeks.

Selective enrichment of bacteria

Bacteria are suspended at a concentration of 10^7-10^8/ml in a defined minimal medium, suitable for the culture of the bacteria, containing 0.02% (w/v) Tween 20. The optimal concentration of bacteria should be determined for different bacteria and antibodies. Beads (0.5 ml), precoated with specific MAb, are added to 10 ml of bacterial suspension and the mixture is gently shaken by hand for up to 2 min, placed in the MPC and washed three times with broth. The 2-min incubation time is critical and it should not be exceeded. The beads are then added to fresh broth and a non-relevant MAb, such as one specific for a viral protein, should be used as a control to monitor non-specific binding. The enriched cultures can then be monitored by fluorescence microscopy or flow cytometry (Lutton *et al.*, 1991) to determine whether there has been enrichment for a particular epitope.

Study of Attachment

Populations with clearly defined surface structures/antigens can be used in studies of attachment. The most commonly used methods involve adherence of bacteria to erythrocytes (haemagglutination), epithelial cells or cells in tissue culture. These methods are often preliminary to determination of the specific attachment mechanism involved. Other, more non-specific, methods of assessing the ability to attach involve determination of the hydrophobicity of the bacterial surface (see Chapter 2).

Surface hydrophobicity

Hydrophobic interactions are considered to play a major role in the association of bacteria with phagocytes and epithelial cells. In our studies (unpublished data) we have used two of the most common methods for determining the surface hydrophobicity of homogeneous populations of *B. fragilis* separated by Percoll density gradient centrifugation.

Adherence to hydrocarbons

This method is based on the principle that, after brief mixing, hydrophobic bacteria will bind to hydrophobic solvents such as xylene or cyclohexane (Rosenberg *et al.*, 1980). Bacteria are centrifuged and washed twice in PUM buffer (g/l, $K_2HPO_4 . 3H_2O$, 22.2; KH_2PO_4, 7.26; $MgSO_4 . 7H_2O$, 0.2; urea, 1.8; pH 7.1). The bacteria are then resuspended in PUM buffer and the $OD_{550\,nm}$ is determined. The bacterial suspension (1 ml) is then added to a series of 10 mm diameter glass test tubes. Volumes of the hydrocarbon, in a range from 50 to 500 µl, are added to each tube. The tubes are incubated at 30°C for 10 min and are then uniformly shaken for 2 min. The hydrocarbon phase is allowed to separate completely before the lower aqueous phase is carefully removed with a Pasteur pipette. A PUM buffer blank is included as a control. The change in $OD_{550\,nm}$ is then determined for each volume of added hydrocarbon, and attachment is expressed as the percentage decrease, which represents the proportion of bacteria excluded from the aqueous phase.

Hydrophobic interaction chromatography

This determines the proportion of a population of bacteria retained on a hydrophobic gel column, and we have modified the methods of Ismaeel *et al.* (1987) and Mozes & Rouxhet (1987). Columns are constructed from Pasteur pipettes plugged with glass wool, loaded with octyl sepharose CL-4B (Pharmacia Biosystems (UK) Ltd, Milton Keynes, UK) to a bed height of 30 mm and equilibrated with an equilibrating solution (ES) of 4 mol/l NaCl in 0.05 mol/l citrate buffer (pH 4.7). Bacteria are washed and resuspended in ES, 100 µl of the bacterial suspension is diluted in 3 ml of ES and the $OD_{555\,nm}$ determined (OD_0, which may be adjusted to 1). The original bacterial suspension (100 µl) is also applied to the column, followed by 3 ml of ES, which is collected and the $OD_{550\,nm}$ determined (OD_1). Distilled water (3 ml), adjusted to the same pH, is then applied to the column, collected and the $OD_{550\,nm}$ also determined (OD_2). The proportion of bacteria retained by the column at high ionic strength R_F is given by $100\ (OD_0 - OD_1)/OD_0$. The proportion of bacteria retained on the column at low ionic strength R_L is given by $100(OD_0 - OD_2)/OD_0$.

A simpler alternative method is to add 5 ml bacterial suspension in distilled water or quarter-strength Ringer's solution to a column and determine the decrease in $OD_{550\,nm}$.

Haemagglutination

Haemagglutination is one of the simplest methods for the determination

of the adherence of bacteria to animal cells. An excellent reference for haemagglutination methods is Old (1985). Two methods can be used, either the static settling or the rocked-tile method. Both techniques are very simple but each has its advantages and disadvantages. It is recommended that both are used to determine haemagglutination, and that a known positive strain is included in each test as a control.

Preparation of erythrocytes and bacterial suspension

Whole blood, treated with either heparin or citrate to prevent coagulation, should be centrifuged at 200*g* for 10 min and the cells washed gently three times in PBS. The cells are then made up in PBS to 2 or 3% packed-cell volume. The erythrocytes can be stored at 4°C for up to 7 days. Blood from as wide a range of species as possible should be used.

Bacteria are centrifuged, washed and resuspended in PBS. A range of different concentrations of bacteria should be used to determine their optimal concentration. It is useful to start at about 1×10^9 bacteria/ml and proceed with doubling dilutions.

Rocked-tile method

Equal volumes of the erythrocyte and bacterial suspension in PBS are mixed on a chilled tile by rocking. Haemagglutination is judged 'by eye' as the formation of granular clumps of erythrocytes within 5 min (Fig. 6.3). We have found that this test can easily be carried out on a microscope slide and haemagglutination viewed as clumping under a low-power objective. To determine whether sugars inhibit haemagglutination, an equal volume of a 2% (w/v) solution of the sugar of interest is added to the erythrocytes before the bacteria.

Static settling method

Equal volumes of erythrocyte and bacterial suspension in PBS are added to round-bottomed wells. The large-size WHO plates are useful because they are large enough for the haemagglutination to be obvious, but microtitre plates can also be used. The stationary plates are incubated for a standard time, either at 4°C or at room temperature. In the absence of haemagglutinating bacteria, the erythrocytes sediment to the bottom of the well under gravity, and tumble into the centre of the well to form a discrete button. In the presence of haemagglutinating bacteria this process is retarded, and the erythrocytes remain in suspension for longer and settle to form a lawn over the bottom of the well. This will also eventually collapse into a button in the

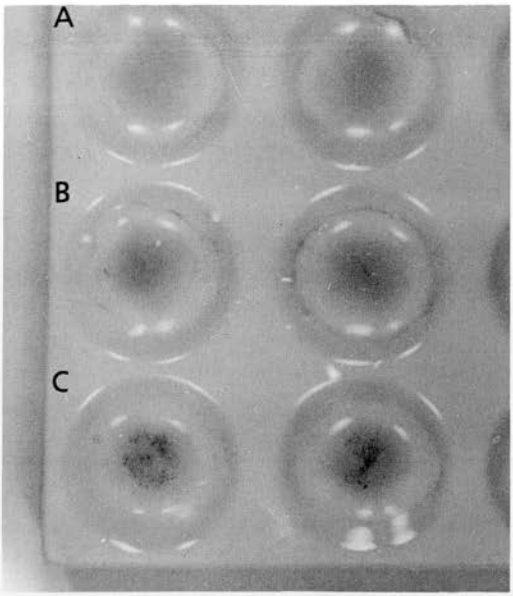

FIG. 6.3. Haemagglutination (HA) of *Bacteroides fragilis* determined by the rocked-tile method: A, capsulate population, HA-negative; B, mixture of capsulate and non-capsulate population, HA-positive; C, non-capsulate (electron-dense layer) population, HA-positive.

centre of the well if the well is left stationary for sufficient time. The extent to which the sedimentation of the erythrocytes is retarded will vary according to the concentration of bacteria and how well they agglutinate the erythrocytes. The incubation time is therefore critical: if the plates are left for too long, positive haemagglutination may be missed. False-positive reactions may occur at very high bacterial concentrations if the bacteria have large capsules that retard the bacterial sedimentation under gravity. The physical size of the bacterium in suspension may prevent the erythrocytes from settling to the centre of the well even after a long incubation period (Patrick *et al.*, 1988). This highlights the need to confirm haemagglutination by both the rocked-tile and the static settling methods.

Where unreproducible haemagglutination results are obtained for a particular bacterial type, this may be due to variation in the proportion of haemagglutinating cells within a single population. Separation of a strain into subpopulations may help to determine whether this is the case. By artificially mixing the subpopulations, the proportion of haemagglutinating bacteria required for a positive result can be determined.

Haemagglutination of bacterial populations grown under different environ-

mental conditions can be compared with the artificially mixed populations. This can be used to determine the selective pressures that favour a higher proportion of haemagglutinating bacteria. To test the artificial mixtures, the haemagglutinating subpopulation (A) is mixed with the non-haemagglutinating subpopulation (B) in the following amounts: (μl) 20A:180B; 40A:160B and so on in 20 μl steps, finishing with 180A:20B. This can conveniently be done directly into the incubation wells with a standard 20 μl dropper pipette. This results in different ratios of the two bacterial types, but the same final bacterial concentration. Erythrocyte suspension (0.2 ml) is then added and the degree of haemagglutination monitored. A gradation in the degree of haemagglutination should be observed (Fig. 6.4).

Adherence to epithelial cells

There is a wide variety of methods for studying the degree to which bacteria adhere to the surface of epithelial cells. The intention here is to illustrate the methods generally adopted in such studies (see Chapter 8), some of which we have used in our studies on *B. fragilis* (unpublished results).

Collection of epithelial cells

Epithelial cells are usually collected from the buccal mucosa of a number of human volunteers by gentle scraping with a blunt and sterile instrument such as a wooden spatula or tongue depresser. They may also be collected from sources such as the urine of healthy females by centrifugation of freshly voided urine. The cells are suspended in PBS. We have also obtained cells from bovine and ovine peritoneal mesothelium by gentle scraping and suspending in PBS.

The cells are usually washed two or three times by centrifugation at 200 *g* for 10 min and resuspended in PBS. The percentage viability of the cells can be determined by dye-exclusion, for example, with trypan blue (Gorman *et al.*, 1986). The concentration of cells in the suspension is adjusted to 10^5 cells/ml after direct counting with a haemocytometer.

The adherence assay

Bacterial cells are washed twice by centrifugation and are resuspended in PBS to a concentration of 10^8 bacteria/ml. This is usually done by adjusting the $OD_{550\,nm}$, based on a previous viable count calibration. The bacterial suspension (250 μl) is then mixed with an equal volume of epithelial cells in an Eppendorf centrifuge tube and incubated at 37°C for 1 or 2 h, which is usually sufficient for the bacteria to attach to the cells. For initial tests it is prudent to determine the

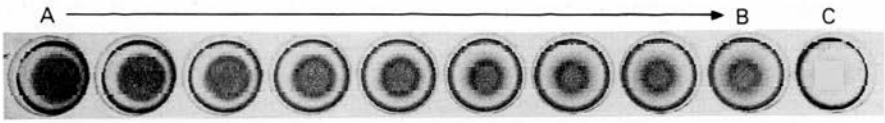

FIG. 6.4. Haemagglutination (HA) of *Bacteroides fragilis* capsulate and non-capsulate populations mixed in a series of proportions: A, nine parts non-capsulate (electron-dense layer) : one part capsulate, strongly HA-positive to B, one part non-capsulate : nine parts capsulate, HA-negative; C, control without bacteria.

optimum incubation time for attachment. The cells are then washed by centrifugation at 200 *g* for 10 min to remove any bacteria not firmly attached. If an Eppendorf centrifuge with a low speed facility is not available, the Eppendorf tubes can be lowered into the tube holder of a bench centrifuge swing-out head with a length of cotton thread. A PBS control without added bacteria should always be included to assess the background level of commensal bacteria adherent to the epithelial cells.

The cells are finally resuspended in 500 μl PBS, smeared onto a microscope slide and fixed with methanol. A variety of stains, from fluorescent acridine orange to Giemsa stain (Sigma Chemical Co (UK) Ltd, Poole, UK), can be used to detect the adherent bacteria. We have used negative phase-contrast with a ×100 oil-immersion objective to observe bacteria without staining. Giemsa stain, which rapidly stains nuclear material in about 45 s, is also satisfactory and can be washed off with methanol. The bacteria stain blue, as does the nucleus of the epithelial cells. Other methods that can be used to determine the attachment of bacterial cells include the use of radioisotope-labelled bacteria (Calderone *et al.*, 1984; Gorman *et al.*, 1987). The adherence assay is carried out essentially as above, except that the bacterial cells are labelled with ^{3}H-leucine. After incubation with the epithelial cells, the mixture is filtered through a membrane filter (8 μm pore size, Millipore (UK) Ltd, Watford, UK), washed several times with PBS, transferred to a tissue solubilizer (e.g. NCS, Amersham International Plc, Amersham, UK) before the addition of scintillant and scintillation counting. The percentage of bacteria attached to the cells is given by:

$$\frac{\text{cpm sample} - \text{cpm background}}{\text{cpm bacterial suspension}} \times 100$$

Data evaluation

Bacteria attached to at least 50 cells and 50 control cells should be counted. The distribution of bacterial attachment to cells may be important in analysing

the data, for example when comparing two sets of data. Most published work assumes that the distribution is normal and hence uses a *t*-test, which is parametric and assumes a normal distribution, to compare the mean and standard deviation from two sets of data. In our experiments with *B. fragilis* (unpublished data), however, and in other published work, it is clear that adherent bacteria are not normally distributed. It would therefore be more appropriate to use a non-parametric and distribution-free statistical analysis, such as the Mann–Whitney U-test. However, there can also be problems with this test because it ranks the data and does not work well if cells have the same number of attached bacteria. The suitability of both these tests is reviewed by Rosenstein *et al.* (1985) and Woolfson *et al.* (1987). In the latter the conclusion was that, despite the non-normal distributions, the Student's *t*-test was probably the more useful method (see Chapter 19).

Adherence to other cells

Experiments similar to those with epithelial cells have been carried out with cultured cells. Another approach is to assess the adherence of bacteria to a monolayer of cells cultured on glass cover-slips (Hartley *et al.*, 1978). Here, the cover-slips (6 × 22 mm) are treated with EDTA (elthylenediamine tetra-acetic acid) and placed into individual glass tubes. They are then seeded with about 10^5 cells (e.g. HeLa cells) and incubated at 37°C for 2 days to produce a confluent monolayer. The coverslips are then washed with two changes in PBS, and the bacterial suspension is added. After further incubation for 30 min, the cells are washed five times in PBS, fixed with methanol, stained with Giemsa and mounted on a microscope slide.

Inhibition of adherence

In order further to characterize the nature of the interaction between the bacteria and the cell surface, substances likely to inhibit attachment specifically can be added to the adherence assay. Such studies often involve the purification of bacterial surface structures involved in adhesion, such as the fimbriae, and modification of the adherence assay. The details of these techniques are beyond the scope of this chapter. A general review of the approaches adopted can be found in Arp (1988).

Addition of sugars

Sugars, such as mannose which inhibits haemagglutination by *E. coli* Type 1 fimbriae (Duguid *et al.*, 1966), may be added to the assay. Haemagglutination that is inhibited by mannose is termed mannose-sensitive, and it is accepted

that the receptor on the erythrocytes contains mannose. Mannose-resistant adhesion may involve other sugar receptors on the cells: for example, K99 fimbriae of enterotoxigenic *E. coli* bind to Neu5Gc(α2-3)Gal(βl-4)Glc-(βl-1) ceramide (Smit *et al.*, 1984) and the P fimbriae of *E. coli*, involved in pyelonephritis, bind to Gal(α1-4)Gal-disaccharide moieties associated with the P blood group antigens. The possibilities are extensive. Generally, sugars of interest are added at about 2% (w/v) to the prepared erythrocytes or epithelial cells before adding the bacteria. If it is desired to determine the host cell receptor for a particular bacterial ligand, reference should be made to Karlson & Stromberg (1986). A general discussion of the importance and diversity of oligosaccharide moieties in mammalian systems is provided by Rademacher *et al.* (1988).

Addition of monoclonal antibodies

Since MAbs bind specifically to target epitopes on bacterial surface structures, they can be a powerful tool for the determination and characterization of surface structures associated with adhesion and their interaction with target cells. It is assumed that binding of the MAb to an epitope on the surface structure of interest will interfere with adherence. This approach has been successfully used for *Porphyromonas* (*Bacteroides*) *gingivalis* fimbriae (Isogai *et al.*, 1988). Before adding a MAb in a haemagglutination or adherence assay, it is necessary further to purify the MAb. This precaution should also be adopted when using monospecific polyclonal antibodies. We have found that serum, including fetal calf serum, added to cell culture media, can cause bacterial clumping and this interferes with the adherence assay. Ascites fluid or cell culture supernatant is centrifuged at 20 000 *g* for 20 min and the resulting supernatant chromatographed on Sephadex G200 (Pharmacia Biosystems (UK) Ltd) with PBS as the elution buffer, and the immunoglobulin — usually IgG — is collected. The MAb is then added to the erythrocyte or cell suspension, before adding bacteria to the adherence assay. This should be done as twofold serial dilutions of the MAb in PBS, usually starting at about 1 mg/ml.

Conclusions

The interaction between the bacterial surface and the surfaces of mammalian cells remains one of the most important steps in the process of pathogenesis. The interactions are often complex and depend on the species of bacterium and type of target cell. It is clear that an approach adopted for one pathogen may be unsuitable for others. Moreover, bacterial surface structures may be subject to environmentally induced variation: surface structures or antigens expressed during an infection may be different from those in laboratory

culture. The definition of bacterial growth conditions is therefore important. In addition, genetically controlled phase variation mechanisms may operate within a population of bacteria in culture. This may result in the heterogeneous expression of adhesion factors within a single population grown under the same environmental conditions. Therefore, the use of a defined homogeneous population of bacteria is also essential. A wide variety of microbiological, immunological, chemical and biochemical techniques has been used to dissect the processes involved in attachment. A concerted attempt to understand these interactions necessarily involves the use of all of these techniques in combination.

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Immune reactions to *Bacteroides fragilis* populations with three different types of capsule in a model of infection

Sheila Patrick,[1] Deborah A. Lutton[1] and Alistair D. Crockard[2]

Author for correspondence: Sheila Patrick. Tel: +44 1232 240503. Fax: +44 1232 438181.

Department of Microbiology and Immunobiology, School of Clinical Medicine, Queen's University of Belfast[1], and Regional Immunology Laboratory[2], Royal Victoria Hospital Site, Grosvenor Road, Belfast BT12 6BN, UK

The survival and growth of populations of the obligately anaerobic pathogenic bacterium *Bacteroides fragilis* enriched for large capsules (LCs), small capsules (SCs) or an electron-dense layer (EDL; non-capsulate by light microscopy) were examined in a mouse model of infection over a minimum period of 20 d. Chambers which allowed the influx of leukocytes, but not the efflux of bacteria, were implanted in the mouse peritoneal cavity. The LC and EDL populations consistently attained viable cell densities of the order of 10^8–10^9 c.f.u. ml^{-1} within 24 h, whereas the SC population did not. However, after 3 d, all three bacterial populations maintained total viable numbers of 10^8–10^9 c.f.u. ml^{-1} within the chambers. LC expression was selected against within 24 h in the model, the populations becoming non-capsulate by light microscopy, whereas in the SC population expression of the SC was retained by approximately 90% of the population. The EDL population remained non-capsulate by light microscopy throughout. Lymphocytes infiltrated the chambers to an equal extent for all three *B. fragilis* populations and at approximately 1000 times higher concentration than chambers which contained only quarter-strength Ringer's solution. The presence of neutrophils within the chambers did not cause a decrease in the total viable bacterial count. Each population elicited antibodies specific for outer-membrane proteins and polysaccharide, as detected by immunoblotting, which cross-reacted with the other populations. Differences were observed in the immunogenicity of the outer-membrane proteins within the three populations. Neutrophils were initially the predominant cell type in the chambers, but as the total leukocyte count increased with incubation time, neutrophils were outnumbered by other leukocytes. Flow cytometric investigations indicated that by day 7 the majority of these leukocytes were B-cells. Bearing in mind the constraints of this model system, it appears that all three populations of *B. fragilis* have the potential for *in vivo* growth and that each elicits an immune reaction.

Keywords: *Bacteroides fragilis*, polysaccharide capsules, immune response

INTRODUCTION

The obligately anaerobic bacteria of the genus *Bacteroides* are numerically the predominant component of the normal commensal faecal flora of the colon in all adult humans, where obligate anaerobes outnumber facultatively anaerobic bacteria by about 1000 to 1 (Willis, 1991). Of the *Bacteroides* species in the faecal flora, *Bacteroides vulgatus* predominates and is estimated to account for up to 45% of the total bacteria present. *Bacteroides fragilis* is less prevalent and estimates of its contribution to the total bacterial faecal flora include 4% (Namavar *et al.*, 1989) and up to 13% (Willis, 1991). However, *B. fragilis* is estimated to account for 42% of the adherent colonic mucosal flora (Namavar *et al.*, 1989). *B. fragilis* is the most commonly encountered species in infection resulting from faecal contamination, whereas *B. vulgatus* and other related *Bacteroides* spp. in the '*B. fragilis*'

Abbreviations: EDL, electron-dense layer; LC, large capsule; RS, Ringer's solution; SC, small capsule.

group are infrequently isolated. Infections from which *B. fragilis* has been isolated include abdominal, pelvic, perianal and vaginal abscesses (Tally & Ho, 1987). Peritonitis, resulting from the leakage of the bowel contents into the peritoneal cavity as a result of, for example, appendicitis or bowel surgery, is a classical example of a *B. fragilis* infection. Although *B. fragilis* and related species within the '*B. fragilis*' group are not members of the normal commensal vaginal flora, they are also responsible for up to half of the upper genital tract, pelvic and uterine infections in women (Duerden, 1991). The majority of *B. fragilis* infections are polymicrobial; for example, *Escherichia coli* and *Streptococcus milleri* are frequently isolated in association with *B. fragilis* (Willis, 1991; Patrick *et al.*, 1995). Although the relationship between these bacterial species may be synergistic, it is generally considered that antibiotic therapy against the anaerobic component of the polymicrobial infection is essential for successful resolution (Rotstein & Meakins, 1990).

The precise virulence determinants of *B. fragilis* remain to be defined; however, surface structures (such as capsular polysaccharides and fimbriae), the release of extracellular enzymes which degrade components of the host tissue (e.g. neuraminidase) and the release of factors which inhibit phagocytic function may all be involved (Patrick, 1993). *B. fragilis* expresses at least three different types of encapsulating surface structure which can be characterized by electron microscopy of ultrathin sections; namely, a large capsule (LC), a small capsule (SC) and an electron-dense layer (EDL). The EDL is visible by electron microscopy but not by light microscopy. Each of these structures may be produced within a single strain. Populations bearing these can be enriched by subculture after Percoll density-gradient centrifugation separation (Patrick & Larkin, 1993). Labelling with monoclonal antibodies specific for surface polysaccharides reveals antigenic variation within these enriched populations (Patrick & Lutton, 1990a).

The relationship of these encapsulating structures to virulence is not clear, but it is possible that the EDL plays a role in attachment to host cells (Patrick, 1988, 1993). The presence of the LC in *B. fragilis* impedes phagocytic uptake and killing in studies of phagocytosis *in vitro* (Reid & Patrick, 1984); however, populations enriched for the LC are rapidly selected against during growth in a mouse model of infection. Within 24 h, LC-expressing cells decrease from 99% of the population to less than 30%, despite the presence of neutrophils (Patrick *et al.*, 1984; Patrick, 1988). In contrast, after 24 h growth in defined medium broth culture, LC-expressing cells constitute more than 90% of the total population. During continuous subculture *in vitro* the population gradually reverts to a mixed capsulate and non-capsulate population over a period of days (S. Patrick, unpublished data). It may be that *in vivo* the bacteria switch from the production of a discrete capsule structure to release of free slime. Surface-associated polysaccharide in the LC population and excreted polysaccharide in the EDL population have common epitopes (Lutton *et al.*, 1991).

In the present study we determine the immune reaction to the LC, SC and EDL populations in an infection localized within chambers implanted in the mouse peritoneal cavity over a minimum period of 20 d. The generation of immunoglobulin specific for both outer-membrane proteins and polysaccharides was demonstrated for each population. The influx of neutrophils and B-lymphocytes into the chambers was observed and not found to affect the total viable bacterial numbers.

METHODS

Bacterial strains. The strain used in this study was *Bacteroides fragilis* NCTC 9343 (National Collection of Type Cultures, Colindale Avenue, London).

***In vitro* culture methods.** Bacteria were incubated in minimal defined medium (MDM) broth (van Tassell & Wilkins, 1978). Cultures were incubated at 37 °C in an atmosphere of $H_2/N_2/CO_2$ (10:80:10, by vol.) in an anaerobic cabinet (MK III Don Whitley Scientific). Identification was confirmed with the API20A system.

Separation and enrichment of bacterial populations. Bacterial populations were enriched for different sizes of encapsulating surface structures by Percoll (Pharmacia) discontinuous density-gradient centrifugation as previously described (Patrick & Larkin, 1993). Populations enriched for the SC by Percoll gradient centrifugation were further enriched, in an anaerobic atmosphere, using immunomagnetic beads (Dynabead M-450 Dynal) coated with anti-mouse immunoglobulin and mouse monoclonal antibody Bf4 (Reid *et al.*, 1987), which is specific for the SC population; this was followed by one subculture in MDM broth. The efficiency of the immunomagnetic bead enrichment method was tested by mixing LC and SC bacteria obtained from Percoll gradient separation in a ratio of 1:1 and enriching from this population with the coated immunomagnetic beads. Following overnight culture in broth it was estimated by light microscopy and negative staining that less than 1% of the population had LCs. The enrichment of the SC population was confirmed by flow cytometric analysis as previously described (Lutton *et al.*, 1991).

Preparation of bacterial outer membranes and polysaccharide. Outer-membrane proteins were prepared as previously described (Patrick & Lutton, 1990b). In brief, bacterial suspensions were rotated 'end-over-end' for 30 min in 3% (w/v) *N*-lauroylsarcosinate (Sarkosyl). After centrifugation in an Eppendorf centrifuge 5414 at 9980 ***g*** the supernatant fraction was discarded and the pellet containing the outer membrane was retained. Polysaccharide was extracted by the proteinase K method (Brown *et al.*, 1989).

SDS-PAGE and immunoblotting. SDS-PAGE was performed on vertical slab gels (8%, w/v) and immunoblotted as previously described (Lutton *et al.*, 1991). The human serum sample was obtained from a patient who was blood-culture positive for *B. fragilis*. The sera and chamber fluids were used at a dilution of 1 in 100.

***In vivo* culture of bacteria.** Chambers were constructed and implanted (two per mouse) in the mouse peritoneal cavity as previously described (Patrick *et al.*, 1984; Lutton, 1991). The chambers were sealed with 3 μm pore membrane filters (Type SSWP, Millipore) which allowed entry of leukocytes but not leakage of bacteria. If filled and sealed chambers were incubated in MDM broth there was no growth in the broth. The bacterial inoculum consisted of a suspension (0·2 ml) of

approximately 5×10^7 c.f.u. ml^{-1} in quarter-strength Ringer's solution (RS) containing 0·5 g L-cysteine l^{-1} (the L-cysteine helps to maintain the viability of *B. fragilis* in RS). The bacterial cultures were not exposed to air at any stage in the preparation of the chambers. All manipulations, from the standardization of the bacterial suspensions by optical density reading to filling and sealing the chambers, were carried out inside an anaerobic cabinet. Chambers were transported to the operating theatre in bottles containing RS inside a sealed anaerobic jar and only removed immediately prior to implantation in the mouse. After implantation of the chambers in the mouse for 1, 3, 7, 14, 20 or 44 d the mice were killed, bled from the heart and the chambers removed. After clotting, the serum was separated and stored at −20 °C. The chamber contents (0·2 ml) were removed and the inside of the chamber washed out vigorously with a further 1·8 ml sterile 0·01 M PBS (0·15 M NaCl, 0·0075 M Na_2HPO_4, 0·0025 M $NaH_2PO_4.2H_2O$), pH 7·4, in 0·2 ml amounts. The data were obtained from four to eight mice, for each sampling day, and a minimum of two independently set-up experiments, with the exception of the initially SC population on days 7, 14 and 44 where two mice were examined. Each of the chambers was examined individually, except where the total leukocyte count was low when the contents of two chambers from the same mouse were pooled for examination of the leukocyte populations. Control chambers containing only RS and RS with Percoll were also examined.

Examination of chamber contents. The total viable bacterial numbers within the chambers were determined by carrying out serial dilutions in RS and plating onto lysed blood agar as previously described (Patrick *et al.*, 1984). All manipulations were carried out inside an anaerobic cabinet. An aliquot (0·2 ml) of the diluted chamber contents was subcultured overnight in MDM broth. Eosin/carbol fuchsin capsule smears of bacteria, taken directly from the chambers and after overnight subculture, were examined by light microscopy (Patrick & Larkin, 1993). The SC expression was also examined by flow cytometric analysis (Lutton *et al.*, 1991).

Heparin (50 µl) was added to 1·5 ml of the diluted chamber contents in PBS which were incubated at 37 °C for 30 min to remove non-specific surface immunoglobulin from the leukocytes. The chamber contents were then centrifuged at 330 ***g*** for 5 min in an MSE Mistral 3000i and the supernatant fraction (i.e. chamber fluid) removed and stored at −20 °C. The cell pellet was washed twice by centrifugation and resuspension in PBS containing foetal calf serum (0·1 %, v/v; FCS). The total viable leukocyte count was determined by microscopy using a haemocytometer counting chamber with the cells suspended in trypan blue (0·08 %, w/v). For differential staining the cells were resuspended at a concentration of 1×10^6 cells ml^{-1} in RPMI1640 medium (Gibco) containing FCS (5 %, v/v) and centrifuged in a cytospin (Shandon Elliot) at 400 r.p.m. for 10 min. The slides were air-dried and either stored at room temperature prior to esterase staining or at −20 °C.

Identification of leukocyte populations. Identification of chamber-infiltrating leukocytes was carried out by morphological, cytochemical and flow cytometric techniques. Preparations from normal mouse peripheral blood and peritoneal washes were used as positive controls for the staining procedures. Morphological analyses were performed on Diff-Quik (Merz-Dade) or haematoxylin-stained preparations. Monocytes/macrophages were identified following α-naphthol staining of leukocyte preparations (Li *et al.*, 1973).

B-lymphocytes were identified by flow cytometric analyses of cell suspensions which had been labelled with the mouse B-lymphocyte marker anti-mouse Ly-5 (B220)-fluorescein-conjugated antibody (Coulter Immunology) as follows. Heparinized chamber contents were incubated at 37 °C for 30 min to remove non-specific surface immunoglobulin, then washed twice by centrifugation in PBS containing bovine serum albumin (2 %, w/v) and incubated with the B-cell marker for 30 min at 4 °C in the dark. The samples were then washed twice as before and finally suspended in PBS containing paraformaldehyde (1 %, v/v) prior to flow cytometric analysis. Heparinized mouse peripheral blood was labelled as above, except that the red blood cells were lysed with Immuno-lyse reagent (Coulter Immunology). Lysis was stopped by the addition of Immuno-lyse fixative (Coulter Immunology). The leukocytes were then washed twice as above and resuspended in PBS/paraformaldehyde. An irrelevant fluorescein-conjugated antibody, anti-human CD4 (Becton Dickinson), was used as a negative control.

Analyses were carried out with an EPICS 5 flow cytometer (Coulter Electronics) equipped with a 5 W argon laser and tuned to 488 nm and operating at a power output of 300 mW at the following setting: forward angle light scatter (FALS) gain, 10; 90° light scatter (LS) detector, 420 V; green fluorescence detector, 1300 V. The lymphocyte population was identified on the basis of size by FALS and 90° LS signals and gated appropriately. Single parameter log integral green fluorescence signals were obtained from the gated population. Five thousand cells were counted in each sample and the percentage of positively staining cells was obtained by subtraction of negative control histograms from test histograms using the instrument's Immuno-programme.

Statistics. The error bars on the graphs represent the confidence limits at 5 % probability ($sigma_n.t$) where n is a minimum of four.

RESULTS

Bacterial viability and leukocyte analyses

Populations of *B. fragilis* initially enriched for the LC, SC and EDL grew in the chambers and attained viable cell densities of over 10^9 c.f.u. ml^{-1} (Fig. 1); however, growth of SC-enriched populations was more erratic during the

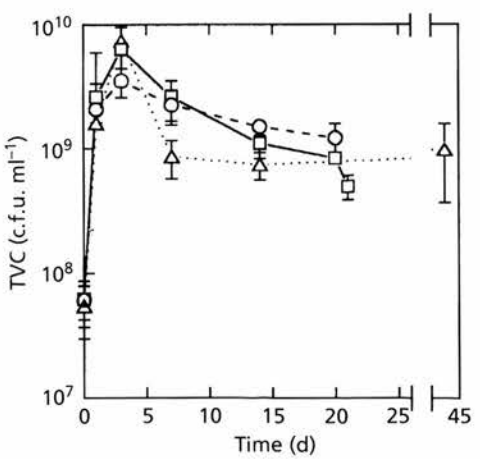

Fig. 1. Total viable bacterial counts (TVC) of *B. fragilis* populations enriched for the LC (--○--), SC (···△···) and EDL (—□—) during incubation in implanted chambers. Error bars represent confidence limits at 5 % probability.

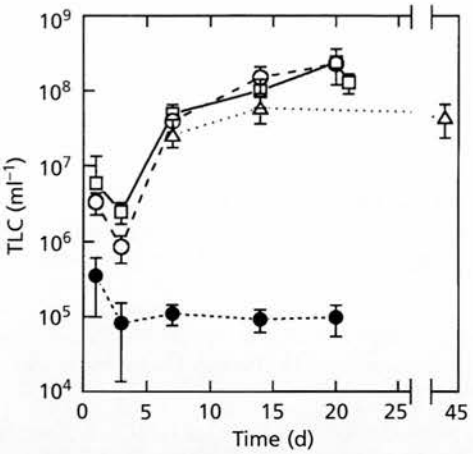

Fig. 2. Total viable leukocyte counts (TLC) in chambers containing only quarter-strength RS with 0·5 g L-cysteine l^{-1} (--●--), or *B. fragilis* enriched for the LC (--○--), SC (···△···) and EDL (—□—) during incubation *in vivo*. Data are not available for the SC population on days 1 and 3. Error bars represent confidence limits at 5% probability.

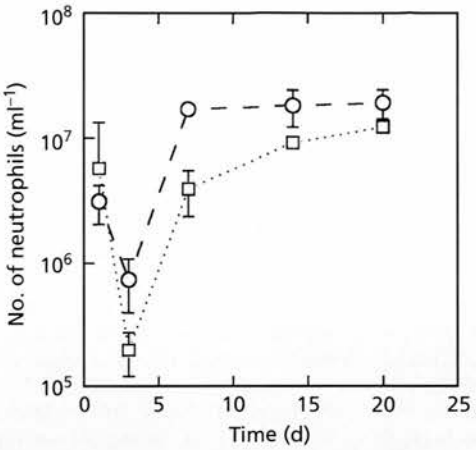

Fig. 3. Numbers of neutrophils in chambers containing *B. fragilis* populations enriched for the LC (○) and EDL (□). Data were obtained from differential microscopical counts and calculated from the total leukocyte count. Data are not available for the SC population. Error bars represent confidence limits at 5% probability.

first day of incubation. Out of a total of 10 mice examined 24 h after chamber implantation, no viable bacteria were detected in both chambers from three of the mice and the total viable bacterial count was less than or equivalent to the starting inoculum in both chambers removed from three other mice. The remainder attained total viable bacterial numbers of greater than 10^9 c.f.u. ml^{-1}. After 3 d incubation, the numbers of viable SC population bacteria in all the mice examined were similar to those of the LC- and EDL-enriched populations. In chambers containing either the LC or EDL populations total viable bacterial counts of between 10^8 and 10^9 c.f.u. ml^{-1} were obtained consistently (Fig. 1). Host material, probably fibrin, gradually built up on the external surface of the chamber membrane filters and by day 7 the chambers were fully encased. The chamber contents also became progressively more viscous during the incubation period. Whether this was due to host- or bacteria-derived material is not known. Observation of the filters revealed that the membrane filters of control chambers without bacteria did not have the same level of deposition of host material, although they did become encased in a thin film, and their contents were less viscous.

The LC-enriched population changed from 99% LC to 97–98% non-capsulate by light microscopy within 24 h and remained at this level for the 20 d period. This decrease in LC-expressing bacteria was evident on direct examination of the chamber contents as well as after one overnight subculture of the chamber contents in MDM broth. The change was therefore not due to environmental modulation. This decrease in the number of LC-expressing bacteria does not occur on subculture directly from the Percoll gradient into MDM broth where 95–100% of the bacteria continue to express the LC. In populations enriched for the SC, an average of 91% (range 80–96%) of bacteria were estimated to express the SC by light microscopy and an average of 96% (range 90–99%) by flow cytometric analysis using monoclonal antibody Bf4, throughout the 44 d incubation period. The rest of the population was non-capsulate by light microscopy, with the exception of day 7, where 1·5% of the population expressed the LC. The EDL population remained non-capsulate by light microscopy when examined directly upon removal from the chamber and also after one subculture of the chamber contents into MDM broth.

The total leukocyte count in the chambers increased progressively with the duration of implantation in the mouse after an initial decrease between day 1 and day 3, and was in excess of that observed in control chambers containing only diluent (quarter-strength RS with 0·5 g L-cysteine l^{-1}) (Fig. 2). To determine if any residual Percoll in the bacterial inocula had an effect, an 80% suspension of Percoll, diluted 1 in 500 in RS, was incubated in the chamber model for 3 and 21 d. This resulted in similar leukocyte infiltration to that observed with chambers containing only RS.

Differential counts performed on Diff-Quik-stained cytospin preparations indicated that neutrophils were the predominant infiltrating cell at day 1 (97% and 94% for chambers containing the EDL and LC populations, respectively). However, by day 14, against a background of increasing total leukocyte count (Fig. 2), neutrophils comprised only 9% (EDL chambers) and 12% (LC chambers) of the total leukocyte count. The numbers of neutrophils in the chambers are illustrated in Fig. 3. Neutrophils containing phagocytosed bacteria were observed by electron microscopy (not illustrated). Classification of the other leukocytes by microscopy proved difficult due to the distorted morphology of the cells. It is

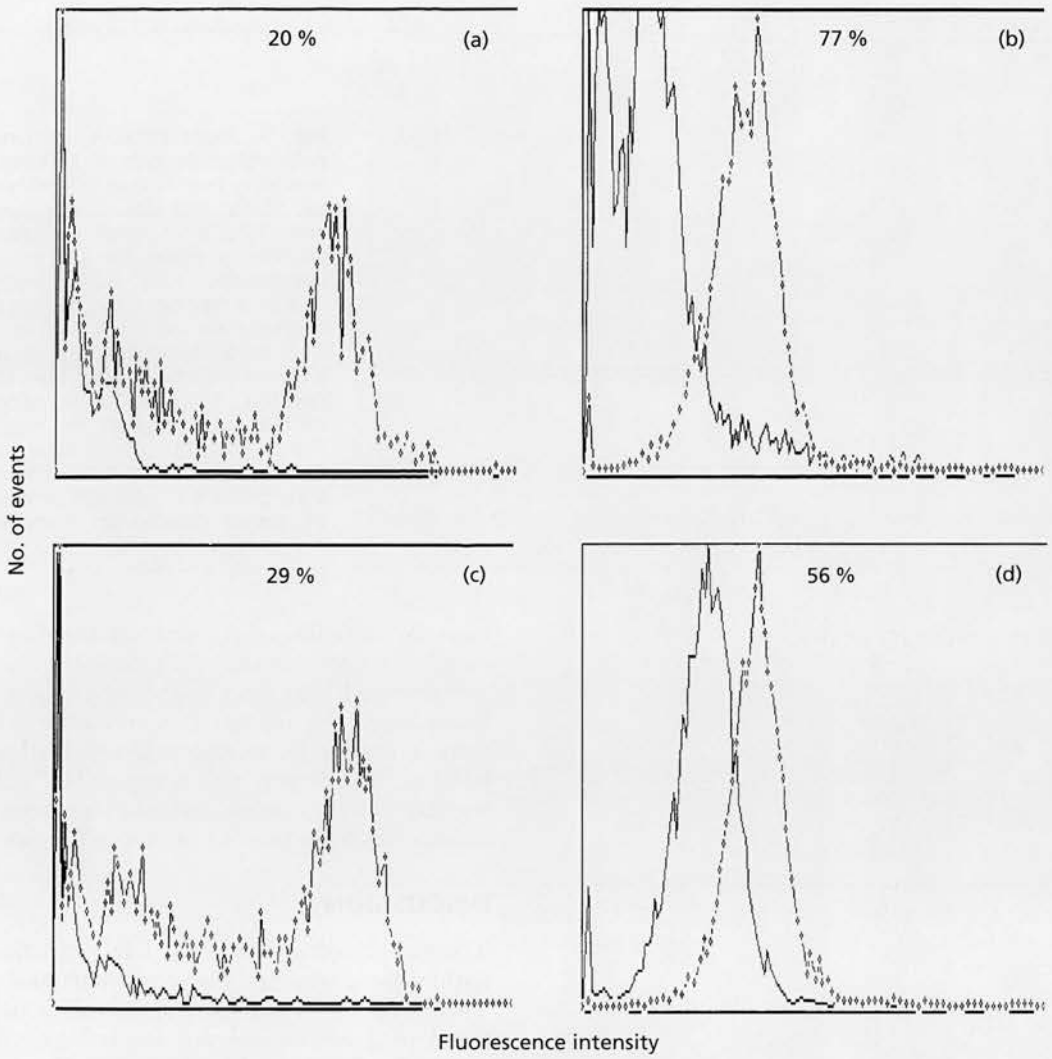

Fig. 4. Representative flow cytometric profiles of Ly-5 (B220) expression by lymphocytes from mice with implanted chambers containing *B. fragilis* enriched for the EDL population after 7 d (a, b) and 21 d (c, d) incubation. (a, c) Peripheral blood; (b, d) chamber contents; dotted line, Ly-5 (B220) positive; solid line, control anti-human T-lymphocyte CD4; X-axis, fluorescence intensity channel number 0–255; Y-axis, number of events scale 0–50. The means of the percentages of Ly-5 (B220)-positive leukocytes are indicated on each graph.

possible that the altered shape of the cells present in the chambers occurred as a result of cellular activation. Cytochemical staining for α-naphthol acetate esterase activity was observed with circulating monocytes and peritoneal macrophages, but not with mononuclear cells in the chambers containing *B. fragilis* or diluent.

Experiments with the EDL population indicated that leukocytes which entered the chamber after the initial influx of neutrophils were B-lymphocytes. Flow cytometric profiles (Fig. 4) of Ly-5 (B220)-labelled cells indicated that on day 7 approximately 75% of the mononuclear cells were positive. Analysis of leukocytes from day 21 samples, however, revealed a high level of labelling of chamber leukocytes with the control antibody. As the proportion of the cells positive for Ly-5 (B220) is calculated relative to labelling with the control antibody, this resulted in an apparent decrease in the numbers of Ly-5 (B220)-positive cells.

Immunoblotting

Sera taken from the mice in which chambers had been implanted were reacted with electroblots of proteinase K (polysaccharide) and Sarkosyl (outer membrane) extracts of each of the LC, SC and EDL populations and detected with alkaline-phosphatase-conjugated anti-mouse IgG (heavy and light chains). Immunoblotting showed a progressive increase in labelling during the time of incubation in the mice. Each of the three populations elicited antibodies reactive with the other populations. Protein and polysaccharide antigens each elicited specific antibody. Selected immunoblots illustrating the reactivity with Sarkosyl extracts are illustrated in Fig. 5. A number

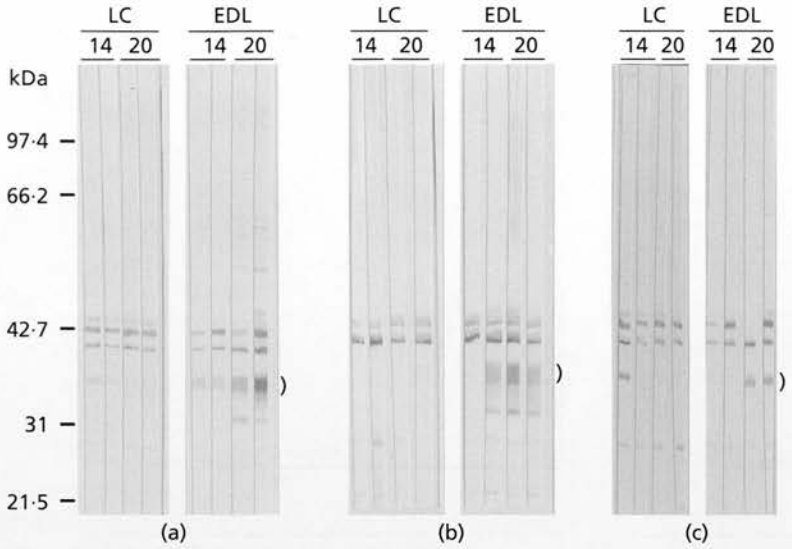

Fig. 5. Representative immunoblots from polyacrylamide gels of Sarkosyl extracts of *in-vitro*-grown *B. fragilis* enriched for the LC (a), SC (b) and EDL (c) populations probed with sera from mice in which chambers containing either the LC- or EDL-enriched populations had been implanted. The numbers represent the duration in days of implantation of chambers in the mouse. Note broad band antigens (brackets) which are not reactive with the LC sera. The reaction of sera from mice with SC-containing chambers is not illustrated. Reaction with sera from mice with chambers containing only quarter-strength RS with 0·5 g cysteine l^{-1} did not produce a pattern of bands. Molecular mass markers are indicated on the left.

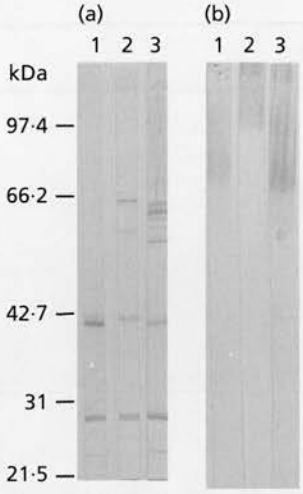

Fig. 6. Immunoblot from polyacrylamide gels of Sarkosyl (a) and proteinase K (b) extracts from *in-vitro*-grown *B. fragilis* enriched for the LC (lane 1), SC (lane 2) and EDL (lane 3) populations probed with a human serum sample from a patient whose blood was culture positive for *B. fragilis* and anti-human IgG alkaline phosphatase conjugate. Molecular mass markers are indicated on the left.

of antigens present in the LC, EDL (Fig. 5) and SC (not illustrated) Sarkosyl extracts in the 31–40 kDa region did not react with the sera from mice implanted with the LC population. In the proteinase K extracts a broad diffuse band in the high molecular mass region of the blot, with an associated fine ladder pattern was observed. This is characteristic of high molecular mass polysaccharide and similar to the pattern previously observed with monoclonal antibody labelling (Reid *et al.*, 1987; Lutton *et al.*, 1991). There was no labelling of the polysaccharide recognized by QUBf5 (Lutton *et al.*, 1991), which has a ladder band-pattern similar to that of the O-antigen of other bacteria. The reactivity of antibodies in chamber fluids, obtained after removal of the bacteria and leukocytes by centrifugation, with Sarkosyl-extracted outer-membrane proteins was also examined. Specific antibody was detected later than in the mouse sera and was of a lower titre. The reactivity of one human serum sample from a patient from whose blood *B. fragilis* had been isolated (Fig. 6) was also compared. The human serum reacted with outer-membrane proteins and polysaccharides from the LC, SC and EDL populations.

DISCUSSION

The growth of the LC- and EDL-enriched populations within the chambers was consistent and reproducible. The exception was the SC population, which on occasion failed to grow or did not reach high viable numbers within the first 24 h of implantation. However, where the SC population did survive, by 3 d the viable numbers were equivalent to those of the other populations and were maintained at that level for up to 44 d. It is possible that the SC population has to adapt in some way for growth in the peritoneal environment, or that some selection is taking place within the population which is not necessary for the initial survival of the LC and EDL populations. The chamber model did not require the addition of, for example, *E. coli* or adjuvant for bacterial growth. *B. fragilis* cells for implantation were suspended in quarter-strength RS with L-cysteine (0·5 g l^{-1}) and not exposed to oxidizing conditions, which reduce total viable numbers. It may be that the dependence of *B. fragilis* on *E. coli* for survival within the peritoneal cavity in a fibrin clot model of infection (Verweij *et al.*, 1991) relates to the metabolic activity of the *E. coli* which keeps the surrounding environment suitably reduced, thus enabling *B. fragilis* to maintain viability. This eventually results in an environment which is sufficiently reduced to allow multiplication of the *B. fragilis*. Synergy between *B. fragilis* and *E. coli*, in the initial stages of infection, may be related to redox potential.

Monitoring of the cellular response to chambers containing bacteria indicated that the host leukocytes entered

the chambers in quantity and that there was no apparent difference between the LC, SC and EDL populations in terms of the numbers of leukocytes which entered the chambers. In concordance with the findings of Verweij *et al.* (1991), neutrophils were readily identified within the chambers as the initially predominant cell type. In their studies of mixed infections of *E. coli* and *B. fragilis* strain BE1, neutrophils were the predominant cell type 2 h after implantation of a fibrin clot in the rat peritoneal cavity. In the present study, labelling with the Ly-5 (B220) mouse B-lymphocyte marker suggests that B-lymphocytes become the predominant cell type within the chamber by day 7, although after 21 d incubation, non-specific labelling with the control antibody made B-lymphocyte quantification difficult. The total viable leukocyte count, however, remained high. The non-specific labelling may be due to immunoglobulin associated with the leukocyte surface membrane or be a consequence of alteration of the surface membrane receptors resulting from cellular activation. Interestingly, the leukocytes apparently remain viable at a redox potential sufficiently low to support the survival and multiplication of *B. fragilis*.

Immunoglobulins specific for protein and polysaccharide antigens were detectable in blood sera, which indicated that each of the LC, EDL and SC populations elicited immunoglobulin which cross-reacted with the other populations. It therefore appears that sufficient bacterial antigens leak from the chambers to elicit antibody production in secondary lymphoid organs and tissues. Immunoglobulin, of a lower titre, was also detectable in the chamber fluids. This may reflect either diffusion of antibody molecules back into the chambers or generation of antibody by the infiltrating B-lymphocytes. The immunoblots showed differences between the immunoglobulin elicited by the LC population when compared with the SC and EDL populations. Certain epitopes in the 31–40 kDa region, although present in the LC population, did not elicit specific immunoglobulin (Fig. 5), whereas the same epitopes in the SC and EDL populations did. It would be of interest to examine the reactivity of these sera against bacterial populations grown under iron-restricted conditions to determine if there was immunoglobulin specific for the 44 kDa iron-repressible outer-membrane protein (Otto *et al.*, 1991). Examination of the single human serum sample indicated that there were immunoglobulins specific for proteins and high molecular mass polysaccharide epitopes in LC, SC and EDL populations. Examination of polysaccharide expression in bacteria present in pus samples from a variety of different body sites by use of monoclonal antibodies indicates that epitopes associated with the LC, SC and EDL may each be present (Patrick *et al.*, 1995).

In spite of the immune reaction, in terms of immunoglobulin production, the influx of leukocytes into the chambers and the presence of quantities of phagocytosed bacteria inside neutrophils, the number of viable bacteria remains high. How this relates to the virulence determinants of the bacteria and how much the activities of the leukocytes are impaired are as yet unresolved questions. Studies on neutrophils removed from *B. fragilis*-induced abscesses indicate that, although the *B. fragilis* cells inside the neutrophils are not killed, the neutrophils are still functional (Finlay-Jones *et al.*, 1991).

This model of infection allows both the growth of bacteria and the immune response to be monitored over a period of weeks. As the bacteria cannot escape from the chamber, but host cells and fluids can enter, this model is a compromise between the open-ended chamber models, from which bacteria may translocate, and the enclosed chambers, which do not allow interaction of the bacteria with host cells (Genco & Arko, 1994). The model is useful, not only because it yields data about fundamental aspects of the immune response to bacterial infection, but it also provides a model for prolonged infection. Whether or not an infection is established in the initial instance may be dependent on bacteria–immune-system interactions in the first few hours after contamination by the bacteria. This has obvious implications for the prevention of post-operative infections (Verweij *et al.*, 1991). However, an abscess, once established, may persist for long periods and infections may recur at the same site. For example, *B. fragilis* has been isolated from persistent pilonidal and perineal abscesses and persistent and recurrent sepsis in the peritoneal cavity (Rotstein & Meakins, 1990). As the bacterial population is limited to the confines of the chamber, any changes in the bacterial population can be related directly to the growth and survival of the bacterial inoculum and not to leakage of the bacteria.

The relationship between this chamber model and biofilm formation on prosthetic devices and implants is also worth consideration, particularly with a view to using the model for the determination of antibiotic activity *in vivo*. The chamber contents are highly viscous and packed with bacteria and leukocytes. The viscosity of the chamber contents may be due to fibrin matrix, as occurs in abscesses (Rotstein, 1992), other host components, bacterial polysaccharide or a mixture of components.

In conclusion, the results of this study indicate that, within the confines of this model, the LC, SC and EDL populations each have the potential for growth *in vivo*, and each elicits an immune reaction. This model therefore allows the detailed study of the interactions of *B. fragilis* with the immune system.

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CLINICAL ANAEROBIC MICROBIOLOGY

Immunological detection of *Bacteroides fragilis* in clinical samples

SHEILA PATRICK, LINDA D. STEWART*, N. DAMANI*, K. G. WILSON, DEBORAH A. LUTTON, M. J. LARKIN†, I. POXTON‡ and R. BROWN‡

*Department of Microbiology and Immunobiology, School of Clinical Medicine, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, * Department of Microbiology, Craigavon Area Hospital, 68 Lurgan Road, Portadown, Co. Armagh BT63 5QQ, † Division of Molecular Biology, School of Biology and Biochemistry, Queen's University of Belfast, Medical Biology Centre, Lisburn Road, Belfast BT7 1NN and ‡ Department of Medical Microbiology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG*

Summary. A monospecific polyclonal antiserum, prepared against *Bacteroides fragilis* common polysaccharide antigen purified by polyacrylamide gel immunoblot detected *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus* and *Prevotella melaninogenica* in pus samples from various anatomical sites by immunofluorescence microscopy of the pus. With standard clinical laboratory culture methods, 36% of 147 samples were positive for one or more of the above bacteria. Of these, *B. fragilis* accounted for 33%. By immunofluorescent labelling of pus with the common antigen antiserum the detection of these bacteria in the samples increased to 50%. All nine of the blood cultures in which *B. fragilis* was detected by culture contained bacteria positive for the common antigen. Immunofluorescent labelling of pus samples with a selection of monoclonal antibodies specific for surface polysaccharides which are known to be antigenically variable in culture *in vitro* and in an animal model of infection showed that these polysaccharides are also variable in natural infection. The results indicate that the common polysaccharide antigen, in contrast to the variable surface polysaccharides, is a suitable target for the immunodetection of *B. fragilis* in clinical samples from a range of anatomical sites.

Introduction

Bacteroides fragilis is the gram-negative obligately anaerobic bacterium most commonly isolated from clinical specimens. It has been isolated, either alone or as a component of mixed infection, from a range of sites in the body, including the peritoneal cavity, genito-urinary tract, blood, lungs and perianal area.[1] The pathogenic contribution of *B. fragilis* in these mixed infections is apparent when the anaerobes are not taken into account in the treatment of the infection and this fails to resolve.[2] *B. fragilis* is a commensal of the gut and can account for up to 13% of the faecal flora whereas other members of the "fragilis group" of *Bacteroides* spp. (*Bacteroides sensu stricto*), such as *B. vulgatus*, predominate. However, in the adherent colonic mucosal flora, *B. fragilis* is the predominant *Bacteroides* spp. with an estimated incidence of *c.* 42%.[3]

As a result of difficulties in maintaining the viability of *B. fragilis* during the transportation of clinical specimens and the time taken to culture the bacteria, direct immunological detection of *B. fragilis* in clinical material by immunofluorescence microscopy has been studied by several workers. These studies have involved the use of a commercially available kit (Fluoretec, Pfizer Diagnostics) based on pooled rabbit polyclonal antisera specific for type strains of *Bacteroides* spp. (*B. fragilis*, *B. vulgatus*, *B. distasonis*, *B. ovatus* and *B. thetaiotaomicron*). The detection of *Bacteroides* spp. by immunofluorescence when compared with culture were reported as 81%,[4] 87%[5] and 97%.[6] The usefulness of this kit as a monitor of faecal contamination of water has also been examined;[7] however, it was not considered to be sufficiently sensitive for the routine monitoring of faecal contamination of disinfected drinking water. The potential diagnostic use of a mouse monoclonal antibody (MAb) specific for the core region of the lipopolysaccharide (LPS) has also been investigated.[8] Although this MAb gave good specific labelling of *B. fragilis* and reacted with 96% of the clinical isolates examined, it labelled only *c.* 10% of the bacteria within a given strain. We have shown previously that

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labelling with MAbs specific for polysaccharide epitopes demonstrates within-strain antigenic variation in *B. fragilis*[9] and it appears that these variable epitopes are immunodominant. Polyclonal antisera raised to whole bacterial cells are also strain specific. This could explain the lack of agreement between the detection of *B. fragilis* by culture and immunofluorescence microscopy in these earlier studies. Therefore, there is a need to identify a non-variable antigen common to *B. fragilis* that could form the basis of an immunodiagnostic test.

In the present study, the suitability of the common polysaccharide antigen described by Poxton and Brown[10] was investigated. This polysaccharide antigen migrates behind the rough form of the LPS and before the smooth form of the LPS on polyacrylamide gel electrophoresis and was reported to be common to seven strains of *B. fragilis* examined by immunoblotting with mono-specific polyclonal antiserum.

This study examined, by immunofluorescence microscopy, the reactivity of a monospecific polyclonal antiserum raised against this polysaccharide antigen with pus samples (and the corresponding pure culture isolates) from a range of different body sites and blood culture samples. The corresponding reactivity of a number of MAbs specific for variable polysaccharide antigens of *B. fragilis* was also examined.

Materials and methods

Bacterial strains

The strains used in this study were *B. fragilis* NCTC 9343 (National Collection of Type Cultures, Colindale Avenue, London), NCTC 10584, ATCC 23745 (American Type Culture Collection, Rockville, MD, USA); *B. vulgatus* NCTC 10583; *B. thetaiotaomicron* NCTC 10582; *B. ovatus* ATCC 8483; *B. distasonis* ATCC 8503; and clinical isolates of *B. fragilis* and other *Bacteroides* spp. obtained from Craigavon Area Hospital, Belfast City Hospital and Royal Victoria Hospital NI (designated LS, JC and including one metronidazole resistant strain, BCH1Mz^r), the Free University, Amsterdam, NL (designated BE) and University of Edinburgh, Scotland (designated GNAB). The *Escherichia coli* and *Staphylococcus aureus* strains used were recent clinical isolates from the bacteriology laboratory at Craigavon Area Hospital, Northern Ireland.

Specimens

The clinical samples were all obtained in Northern Ireland. The majority came from Craigavon Area Hospital, Craigavon; however, a few were also obtained from South Tyrone Hospital, Tyrone, Daisy Hill Hospital, Newry and the Royal Victoria Hospital, Belfast.

Ninety-eight pus samples (Study 1) which had been sent to the laboratories for routine diagnostic testing were examined in detail and information concerning treatment was recorded from the patient's case history where available. These samples were subject to routine laboratory culture techniques (as detailed below) and examined by immunofluorescence microscopy for their reactivity with rabbit polyclonal antiserum to *B. fragilis* common polysaccharide antigen (CAg antiserum) and seven mouse MAbs specific for *B. fragilis* as detailed below. The pure culture isolates of *B. fragilis*, other *Bacteroides* spp. and *Prevotella melaninogenica* obtained from these samples were also examined by immunofluorescence microscopy for their reactivity with the CAg antiserum and the seven MAbs. A further 49 pus samples (Study 2) were examined for the presence or absence of *B. fragilis* by routine diagnostic methods and their reactivity with the CAg antiserum by immunofluorescence microscopy.

Ten blood culture bottles of which nine were culture-positive for *Bacteroides* spp. were examined for their reactivity with the CAg antiserum and three of these were also examined with the MAbs.

The pus samples were obtained by surgical drainage or aspiration and were placed in sterile bottles in volumes of 1–20 ml. The pus samples were either plated out directly (as detailed below) or inoculated into Brain Heart Infusion Broth (Unipath) for 24 h. Blood culture bottles (Roche Diagnostic System) were inoculated with 10 ml of the patient's blood and incubated at 37°C aerobically and anaerobically for a total of 7 days. The cultures were examined by eye for bacterial growth three times a day for the first 48 h and once a day for the next 5 days. If growth was apparent in the anaerobic blood culture bottle, a sample was seeded to anaerobic blood agar and the identification procedures performed as detailed below.

Identification by culture

Samples were routinely plated on the following agar media: horse blood agar (BA; Unipath); anaerobic horse blood agar (ABA; Gibco); colistin-nalidixic acid agar (CNA; Unipath); anaerobic blood agar plus gentamicin 50 μg/ml (ABA + GM). After inoculation, antibiotic disks containing penicillin (1 unit) and gentamicin (10 μg) were applied to the BA and CNA plates and the plates were incubated at 37°C in an aerobic atmosphere with CO_2 10%. Disks containing penicillin (1 unit) and metronidazole (5 μg) were applied to the ABA + GM plate and gentamicin (10 μg), penicillin (1 unit) and metronidazole (5 μg) to the ABA plate. Cultures were incubated at 37°C in an atmosphere of H_2 10%, N_2 80% and CO_2 10% in an anaerobic cabinet (Forma Scientific).

The aerobic plates were examined for colonies after 24 and 48 h and the isolates were gram-stained. Coliform isolates were identified with the API20E system (bioMérieux), *S. aureus* with Staphaurex (Murex Diagnostic), and *Streptococcus* spp. and *Enterococcus* spp. with the Lancefield serotyping kit Streptex (Murex Diagnostic). Streptococci which

could not be classified with the Streptex kit were identified with the API20 STREP (bioMérieux). Small pin head colonies with a characteristic sweet smell that were identified as Group A, C or F, or were otherwise unidentifiable, were classified as *Str. milleri*.

The anaerobic plates were examined after at least 48 h and metronidazole-sensitive organisms were gram-stained. Single colonies were re-streaked on ABA and after 24 h the following tests were performed with the pure cultures: API20A (bioMérieux), ATB 32A (bioMérieux) and the Mastring ID 8 (Mast Laboratories).

Pure culture in brain heart infusion broth with glycerol 10%, pus samples and blood cultures were stored in liquid nitrogen for future examination.

Preparation of antiserum

Monospecific polyclonal antiserum was raised to *B. fragilis* NCTC 9343 as described previously. Briefly, the monospecific polyclonal antiserum described by Poxton and Brown[10] was used to identify the common polysaccharide antigen on guide strips of nitrocellulose after SDS-PAGE and immunoblotting. Small pieces of nitrocellulose containing the common antigen were cut from the unlabelled part of the nitrocellulose and dissolved in dimethylsulphoxide. This was mixed with an equal volume of Freund's complete adjuvant (Difco) and 0·1-ml amounts were inoculated subcutaneously at four sites on the back of a New Zealand White rabbit. Subsequently, the antigen in Freund's incomplete adjuvant was inoculated four times at *c.* 2-week intervals. A further two inoculations of antigen in PBS were made at approximately monthly intervals and the rabbit was bled after each booster dose. Antisera were tested by both immunofluorescence microscopy and immunoblotting.

Immunofluorescence microscopy

Samples (10 μl) from blood culture bottles or pus were applied to one well of a multi-well slide (Flow Laboratories). Four doubling dilutions in phosphate-buffered saline (PBS) were made along the length of the slide. For pure bacterial cultures, a suspension of *c.* 10^7 bacteria/ml in PBS (30 μl) was applied to the slide. A duplicate series of dilutions of each sample was made on the same slide. The slides were air-dried and then fixed in methanol 100% for 10 min at −20°C. Slides with pus or blood culture samples were blocked with undiluted human serum for 10 min. For single labelling, the slides were incubated with either undiluted murine MAb supernates or polyclonal rabbit antiserum diluted 1 in 100 in PBS for 45 min, washed in PBS and then incubated for 45 min with sheep anti-rabbit fluorescein conjugate (Sigma) diluted 1 in 100 in PBS. After a final wash, the slides were examined with a Leitz fluorescence microscope. For dual labelling, the slides were incubated with MAb supernates, washed, incubated with polyclonal rabbit antiserum, washed and incubated with sheep anti-rabbit fluorescein and goat anti-mouse rhodamine diluted 1 in 100 before a final wash.[11] To estimate the sensitivity of the labelling, Evan's Blue (Gurr; 0·05% w/v) was included during incubation with the conjugate. The slides were mounted with glycerol PBS containing an anti-photobleaching agent (Citifluor; Agar Scientific Ltd, Essex). The proportion of bacteria labelled with the antisera was determined by eye and confirmed by photographing the same field of view with filters suitable for viewing the green fluorescence of the fluorescein followed by filters suitable for viewing the red fluorescence of the Evan's blue staining with a fluorescence microscope. The percentage of bacteria labelled with the antisera was estimated by counting the total number of bacteria in each of the photographs. A minimum of 200 bacteria were counted for each estimation. Similarly, where samples were doubly labelled with a MAb and polyclonal antiserum, fields of view were photographed with filters suitable for viewing either the fluorescein (green) or rhodamine (red) dyes. Double-colour photographic exposure of the same field with the two different filter types sequentially were also examined.

Clinical samples were scored positive by immunofluorescence if at least three positive bacterial cells were observed in a sample after extensive examination of the micro-well slide. Each slide was examined for *c.* 10 min. The intensity of fluorescence was also noted.

Results

Sensitivity of labelling with the polyclonal antiserum to CAg

A number of different strains of *B. fragilis* and related bacteria were examined for their reactivity with rabbit polyclonal anti-serum specific for CAg by immunofluorescence microscopy. The total number of bacteria was estimated microscopically after being stained with Evan's blue. All the strains of *B. fragilis* examined were positive for CAg and between 80 and 100% of the bacteria were labelled with the polyclonal antiserum (table I). *B. ovatus* and *B. thetaiotaomicron*

Table I. Sensitivity of labelling of *B. fragilis* strains with the antiserum to the CAg

Strain no.	Bacteria positive (%)	Strain no.	Bacteria positive (%)
NCTC 9343		LS54*	99
EDL population	103	LS66*	98
SC population	95	LS67*	97
LC population	92	BE1	80
NCTC 10584	99	BE3	89
ATCC 23745	96	JC6	98
GNAB 4	86	JC15	100
GNAB 82	96	JC17	98
GNAB 92	98	JC19	100
BCH1 Mz^r	100		

EDL, electron dense layer; SC, small capsule; LC, large capsule.
*Clinical isolates from the current study.

Table II. Detection of *Bacteroides* spp. and related genera in pus samples by culture and immunolabelling with antiserum to CAg (Study 1)

Source of specimen	Number of samples received	Number culture-positive for		CAg positive, *Bacteroides* and *Prevotella* culture-negative
		*B. fragilis**	Other *Bacteroides* and *Prevotella* spp.	
Perianal abscess	15	11	0	2
Abdominal abscess	10	4	1	2
Pilonidal abscess	9	2	5	1
Bartholin's abscess	6	1	0	0
Ischiorectal abscess	4	2	1	1
Vaginal abscess	1	1	0	0
Diverticular abscess	1	1	0	0
Groin abscess	3	1	0	2
Groin neoplasm abscess	1	1	0	0
Colostomy	1	1	0	0
Abscess at pacemaker	1	0	0	0
Haematoma	1	0	0	0
Skin wounds	31	0	0	2
Pleural fluid	2	0	0	1
Gall bladder abscess	2	0	0	1
Liver abscess	3	0	0	0
Brain abscess	2	0	0	0
Pancreatic abscess	1	0	0	0
Peritoneal fluid	1	0	0	0
Parotid abscess	1	0	0	0
Subphrenic abscess	2	0	0	0
Total	98	25	7	12

*All these samples were positive when directly labelled with the CAg antiserum.

Table IIIA. Bacteria isolated from *B. fragilis* culture-positive pus samples

Organisms isolated	Abscess site
B. fragilis	Perianal (2), colostomy (1)
B. fragilis, *E. coli*	Perianal (2), ischiorectal (1)
B. fragilis, *Str. milleri*	Abdominal (1), perianal (2), groin (1), pilonidal (1)
B. fragilis, *Str. milleri*, *E. coli*	Perianal (3), abdominal (1), vaginal (1), ischiorectal (1)
B. fragilis, *Str. agalactiae*	Perianal (1), pilonidal (1)
B. fragilis, *Str. viridans*	Abdominal (1)
B. fragilis, *E. coli*, *Str. milleri*, *Str. agalactiae*	Bartholin's (1)
B. fragilis, *E. coli*, *Str. agalactiae*	Diverticular (1), groin neoplasm (1)
B. fragilis, Group C streptococci	Perianal (1)
B. fragilis, *P. melaninogenica*	Abdominal (1)

Table IIIB. Bacteria isolated from *Bacteroides* spp. and *Prevotella* spp. culture-positive pus samples

Organisms isolated	Abscess site
P. melaninogenica	Abdominal wound (1)
P. melaninogenica, *Peptostreptococcus* sp.	Pilonidal (1)
P. melaninogenica, *E. coli*, *Str. milleri*	Pilonidal (2)
B. thetaiotaomicron, *E. coli*, *Str. milleri*, *Proteus mirabilis*	Ischiorectal (1)
Bacteroides sp., *Str. milleri*	Pilonidal (1)
Bacteroides sp., *Str. milleri*, *Peptostreptococcus* sp.	Pilonidal (1)

Table IV. Bacteria isolated on repeat culture from CAg-positive but *Bacteroides* and *Prevotella* culture-negative pus samples

Organisms initially isolated	Abscess site
None	Buttock (skin wound)*†, labial (skin wound), abdominal†
E. coli	Gall bladder, pilonidal†, groin, perianal, abdominal*†
Str. milleri	Pleural fluid, ischiorectal*†
E. coli, *Str. milleri*	Perianal*
S. aureus, *Str. pyogenes*	Groin†

**B. fragilis* isolated after re-culture of the sample.
†Positive with one or more *B. fragilis*-specific MAb (see table IX).

were labelled with lower intensity but similar sensitivity to *B. fragilis*, and *B. distasonis* gave negative results.

Detection of Bacteroides *spp. in pus samples*

The source of the 98 pus samples examined in Study 1, the culture results for *Bacteroides* spp. and related genera and the reactivity with the CAg antiserum by immunofluorescence microscopy are shown in table II. The bacteria isolated from samples positive for *B. fragilis*, *Bacteroides* spp. and the related genus *Prevotella* by initial culture are shown in tables IIIA and IIIB respectively. Pus samples that were culture-negative for *Bacteroides* and *Prevotella* spp., but positive by immunofluorescence with the CAg antiserum are detailed in table IV. These 12 specimens which were negative for *Bacteroides* and *Prevotella*

Table V. Detection of *B. fragilis* in pus samples by both culture and immunolabelling with antiserum to the CAg. (Study 2)

Source of specimen	Number of samples received	Number culture-positive for *B. fragilis**	CAg-positive, *Bacteroides* culture-negative
Perianal abscess	9	9	0
Pilonidal abscess	8	4	2
Unspecified pus	7	3	1
Abdominal abscess	4	2	1
Scrotum abscess	4	1	2
Pelvic abscess	2	0	2
Mastoid maxillary sinus	3	0	2
Suprapubic abscess	1	1	0
Pleural cavity	1	1	0
Hernia wound abscess	1	0	1
Pouch of Douglas	1	0	0
Neck abscess	1	0	0
Quinsy	1	0	0
Wound abscess	1	0	0
Abdominal drain fluid	1	0	0
Tibial abscess	1	0	0
Breast abscess	1	0	0
Back abscess	1	0	0
Gall bladder abscess	1	0	0
Total	49	21	11

*All except one of these samples were also positive when labelled with the CAg antiserum.

spp. on initial routine testing in the laboratory, but immunofluorescence-positive with the CAg antiserum, were retrieved from storage in liquid nitrogen and re-cultured. Of these, *B. fragilis* was isolated from four (table IV).

Of a further 49 pus samples examined in Study 2, 21 were positive by culture for *B. fragilis*. Eleven of the culture-negative samples were positive by immunofluorescence with CAg antiserum (table V). Immunofluorescence labelling of a typical pus sample is illustrated in fig. 1. With the exception of one sample in Study 2, if a sample was positive for *B. fragilis* on culture it was also positive by immunofluorescence with the CAg antiserum. Of the samples positive for *Bacteroides* spp. or *P. melaninogenica*, or both, only one (no. 3), which was positive for an unidentified *Bacteroides* sp., was negative by immunofluorescence.

In total, 36% of the pus samples were positive for *B. fragilis* and related bacteria by culture and 50% were positive by immunofluorescence microscopy with the CAg antiserum.

Investigation of the patients' case histories

The antibiotic treatment recorded in the case histories of patients whose specimens were either CAg antiserum- or culture-positive for *B. fragilis* is given in table VI. Although metronidazole was prescribed before drainage of the abscesses, samples nos. 11 and 15 were still positive for *B. fragilis* on culture. One sample (no. 68) was culture-negative and the antibiotic treatment was changed to co-fluampicil; however, the current study indicated that this sample was CAg-positive. Examination of the case histories also revealed that four patients had recurrent infections at the same anatomical site (table VII).

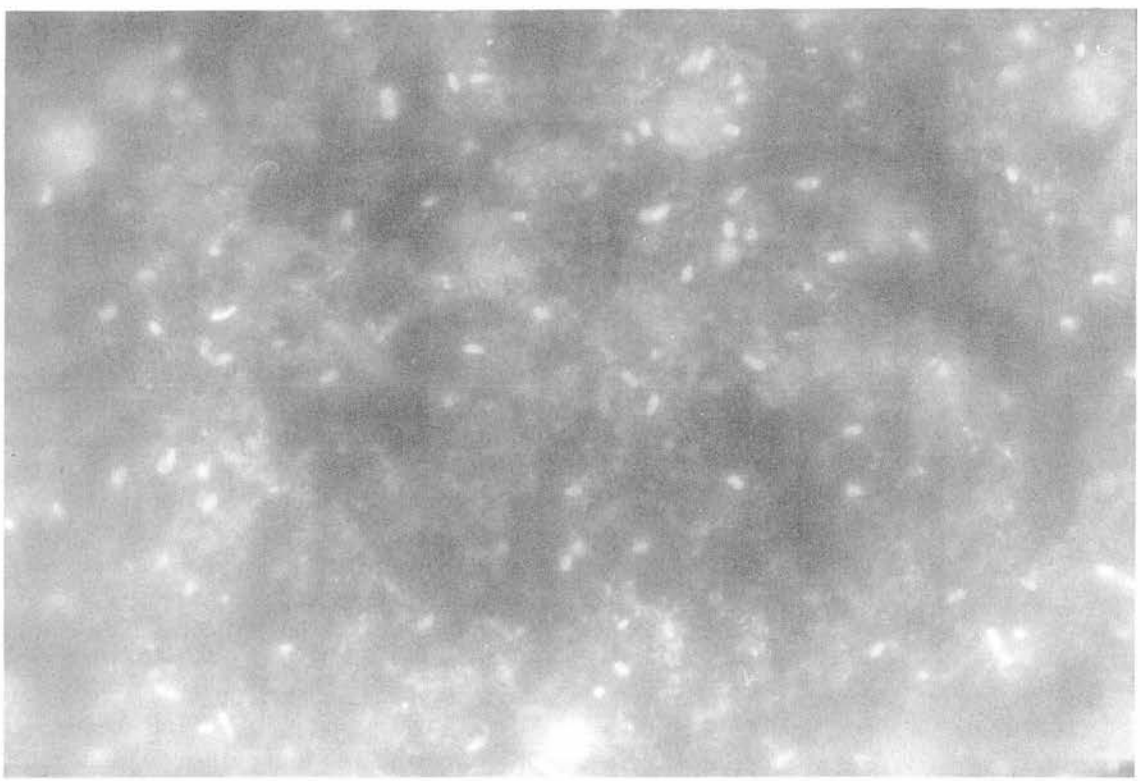

Fig. 1. Micrograph of pus from a groin neoplasm labelled with rabbit antiserum to CAg and anti-rabbit immunoglobulin fluorescein conjugate (× 100).

Table VI. Antibiotics* administered to patients with either *B. fragilis* and related bacteria culture- and CAg-positive (A) or only CAg-positive (B) pus samples. (Study 1)

Sample no.	Antibiotic treatment	
	before drainage	after drainage
A		
4	Co-fluampicil	NI
11	Cefuroxime and metronidazole	Cefuroxime and metronidazole
14	Co-fluampicil	NI
15	Gentamicin and metronidazole	Gentamicin and metronidazole
18	NI	Ampicillin and metronidazole
23	Co-fluampicil	NI
56	Flucloxacillin	NI
66	Metronidazole and cefuroxime	Metronidazole and cefuroxime
73	NI	Erythromycin
75	Amoxycillin	NI
91	Flucloxacillin	NI
96	Co-fluampicil	NI
B		
28	NI	Gentamicin
68	Co-amoxiclav and metronidazole	Co-fluampicil
70	Co-fluampicil	NI
71	Co-fluampicil	NI

NI, not indicated.
*Information obtained from patients' case histories, where available.

Table VII. Bacteria isolated from successive samples obtained from the same patient* based on information in patients' case histories

Sample no.	Abscess site	Date	Culture result
23	Ischiorectal	14.02.89	**B. fragilis*, *Str. milleri*
		12.08.90	*B. fragilis*, *Str. milleri*
70	Pilonidal	29.11.89	**Str. milleri*
		28.05.90	*Str. milleri*, *B. fragilis*
80	Ischiorectal/perianal	14.07.89	No growth
		04.04.90	*Str. milleri*, *B. fragilis*, *P. melaninogenica*
		30.04.90	**Str. milleri*, *B. fragilis*
		24.08.90	No growth
		12.03.91	*Str. milleri*, *B. fragilis*, *P. melaninogenica*
95	Ischiorectal/perianal	29.09.90	**Str. milleri*, *B. fragilis*, *E. coli*
		23.12.87	*Str. milleri*, *B. fragilis*, *E. coli*

*Denotes pus sample examined in Study 1.

Detection of B. fragilis from blood culture bottles

Nine blood culture bottles from which *B. fragilis* was isolated, and one from which *B. distasonis* was isolated, were examined for their reactivity with the CAg antiserum. All nine cultures positive for *B. fragilis* were also positive by immunofluorescence with the CAg antiserum; however, the *B. distasonis*-positive blood culture bottle was CAg-negative. Fig. 2 illustrates typical labelling of a blood culture sample.

Reactivity of B. fragilis clinical samples and pure cultures with murine MAbs

Twenty-five pus samples (from Study 1) and three blood culture bottles, which were all CAg positive, were examined by immunofluorescence microscopy for their reactivity with six murine MAbs specific for the surface polysaccharides of *B. fragilis* associated with the large capsule and electron-dense layer populations. The corresponding pure culture isolated was also examined in each case. MAb QUBf5 is specific for the polysaccharide which forms on PAGE and immunoblotting a pattern similar to that of the O-antigen of other bacterial species. The other MAbs are specific for higher molecular mass polysaccharide associated with the large capsule and electron-dense layer populations of *B. fragilis*.[9]

Four of the pus samples did not react with any of the MAbs when examined directly and the rest reacted with a variable selection of the MAbs. All of the pure culture isolates of *B. fragilis* obtained from these samples were positive for at least one of the MAbs

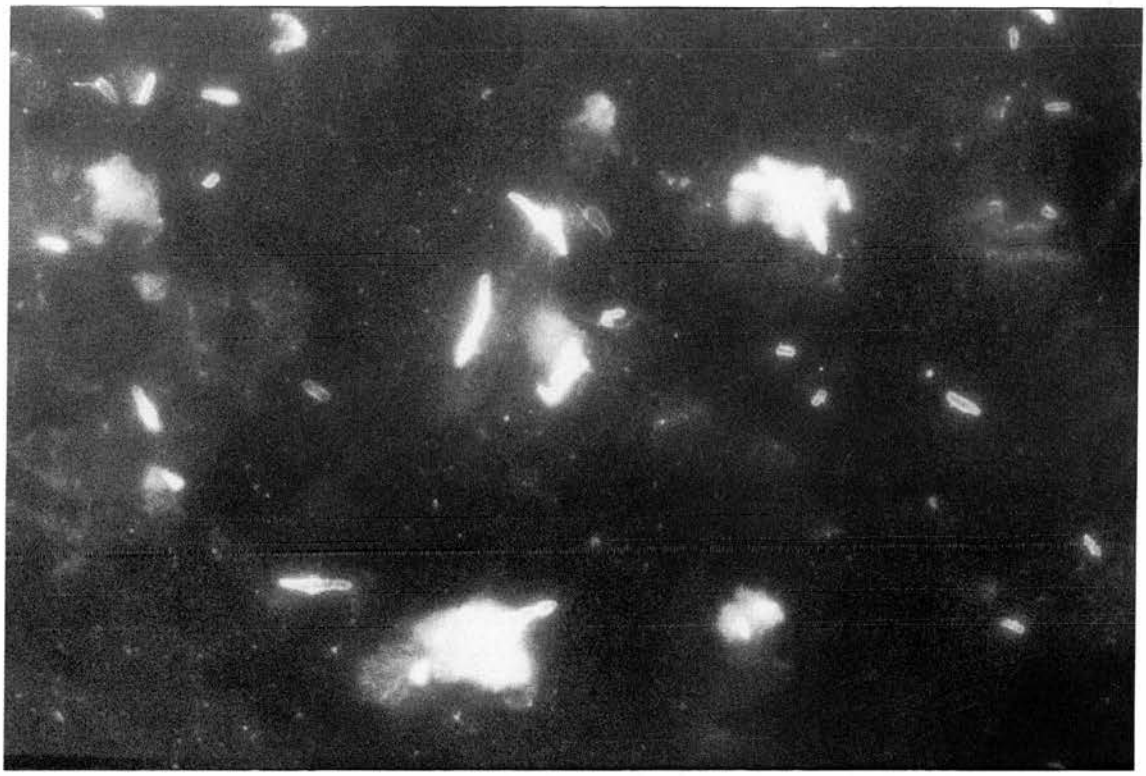

Fig. 2. Micrograph of blood culture labelled with rabbit antiserum to CAg and anti-rabbit immunoglobulin fluorescein conjugate (× 100).

(table VIII). There was no apparent relationship between the site of origin of the sample and the MAb labelling. Double labelling with both the CAg antiserum and the MAbs confirmed that the MAbs label a varying proportion of the *B. fragilis* cells present within a pure culture isolate. This was also the case when pus and blood culture samples were examined directly, without prior culture. Fig. 3 illustrates double labelling of a blood culture. MAb QUBf11, which reacted with all of the pure cultures and 24 of the 28 samples, labelled only 7–15% of the bacterial population. Labelling also indicated that outer membrane vesicles were present in the pus samples.

Examination of the samples that were culture negative for *B. fragilis* and related bacteria, but were positive with the CAg antiserum, indicated that seven of 12 of these samples also reacted with at least one of the MAbs (table IX). Samples from which *Bacteroides* spp. (other than *B. fragilis*) or *P. melaninogenica*, or both, were cultured and which were positive with the CAg antiserum (table IIIB), were negative with all the MAbs. This suggests that the polysaccharides recognised by these MAbs are specific to *B. fragilis*.

Sixteen of the pure culture isolates from Study 1 were examined for their reactivity with a MAb (Bf4) specific for a high molecular mass polysaccharide associated with the small capsule population of *B. fragilis*.[12] An estimated 1%, or less, of the bacteria within these populations were labelled with the MAb. Similarly, the proportion of the bacteria positive with the CAg antiserum, which were also positive with MAb Bf4 when pus samples were examined directly, was $\leqslant 1\%$.

Discussion

The results confirm that the heterogeneous labelling pattern of MAbs specific for high molecular mass polysaccharides of *B. fragilis*, previously observed in culture collection strains and in bacteria grown in a mouse model of peritoneal infection,[13] also occurred in the recent clinical isolates of *B. fragilis* obtained in the study. Variable labelling was also apparent when pus samples were examined directly, without prior culture. Polysaccharides associated with the large capsule, small capsule and electron-dense layer populations[9,12] were all detected in the pus samples with the MAbs. This indicates that antigenic variation in the polysaccharides of *B. fragilis* is apparent during the course of natural infection and raises the possibility that antigenic variation of surface polysaccharides is related to the virulence of *B. fragilis*. Interestingly, there was no obvious relationship between the labelling pattern of the MAbs and a particular site of infection. It is possible that variable antigens are the immunodominant antigens in *B. fragilis* as inoculation with whole cells produces antisera that do not label all the bacterial cells in other strains[14,15] and are thus too insensitive for use as a diagnostic test for the presence of *B. fragilis*. Furthermore, all the polysaccharide-specific MAbs that were raised with whole bacteria as an inoculum were specific for the antigenically variable polysaccharides which are not expressed by all the bacteria within a given population of *B. fragilis*.[16–18] It is possible that these immunodominant variable polysaccharides mask the common polysaccharide. The unsuitability of the variable polysaccharides as targets

Table VIII. Reactivity of clinical samples (S) and the corresponding pure culture isolate (C) of *B. fragilis* with MAbs specific for *B. fragilis* NCTC 9343 surface polysaccharides

Site and sample no.	Reaction with MAb QUBf											
	5		7		8		9		10		11	
	C	S	C	S	C	S	C	S	C	S	C	S
Perianal/ischiorectal abscess												
14	+	+	+	+	−	−	+	−	+	+	+	+
16	−	−	−	−	−	−	+	−	+	−	+	−
23	−	−	−	−	−	−	−	−	−	−	+	+
26	−	−	−	−	−	−	−	−	+	−	+	−
52	−	−	−	−	+	+	−	−	−	−	+	+
54	+	+	+	+	+	+	−	−	+	−	+	+
61	−	−	−	−	+	+	−	−	+	+	+	+
67	+	+	+	+	+	+	−	−	−	−	+	+
75	−	−	+	+	+	+	−	−	−	−	+	+
80	+	+	+	+	+	+	+	+	+	+	+	+
87	−	−	−	−	+	+	−	−	+	+	+	+
92	+	+	−	−	+	+	−	−	+	+	+	+
95	+	+	−	−	+	+	−	−	+	+	+	+
Abdominal abscess												
11	−	−	−	−	−	−	+	−	+	−	+	+
13	−	−	−	−	−	−	−	−	−	−	+	−
66	+	+	+	−	−	−	+	+	+	−	+	+
89	−	−	+	+	+	+	−	−	−	−	+	+
Pilonidal abscess												
56	+	+	−	−	−	−	−	−	+	+	+	+
91	−	−	−	−	+	+	−	−	+	+	+	+
Vaginal/Bartholin's abscess												
18	+	+	+	+	+	+	−	−	−	−	+	+
76	−	−	−	−	−	−	−	−	+	+	+	+
Diverticular abscess												
84	+	+	−	−	−	−	+	+	+	+	+	+
Groin abscess												
73	−	−	−	−	+	+	−	−	+	+	+	+
Colostomy												
15	−	−	−	−	−	−	−	−	−	−	+	−
Groin neoplasm												
27	+	+	−	−	+	+	+	+	+	+	+	+
Blood culture												
39	+	+	+	+	+	+	−	−	−	−	+	+
55	+	−	−	−	+	+	−	−	+	+	+	+
98	−	−	−	−	+	+	−	−	−	−	+	+

C, pure culture; S, pus sample.

for the immunological detection of *B. fragilis* in clinical samples is emphasised. The sensitivity of labelling is not sufficient to allow detection of *B. fragilis* in all instances. It is unlikely that even a mixture of these MAbs could be used effectively as some samples and isolates reacted with only one MAb.

In contrast, a monospecific polyclonal antiserum specific for the CAg of *B. fragilis*[10] labelled between 80 and 100% of the bacteria within 16 strains of *B. fragilis* obtained from culture collections and isolates from Northern Ireland, Edinburgh and Amsterdam, and also populations of strain NCTC 9343 enriched for the large capsule, small capsule or electron-dense layer. The antiserum recognised *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus* and *P. melaninogenica* but not *B. vulgatus* or *B. distasonis*. Why this polysaccharide is common to some species of *Bacteroides* and *Prevotella* but not other species of *Bacteroides* is unclear. Interestingly, there is evidence for the horizontal transfer of genes between *Bacteroides* and *Prevotella* spp.[19] By labelling bacteria directly in pus samples, the detection of these species was increased above that obtained by culture only. The culture-negative immunofluorescence microscopy-positive samples are unlikely to represent false positives, as (i) four of these yielded viable *B. fragilis* when the isolation procedures were performed a second time from the stored pus samples, with particular care to minimise the exposure of the material to air and not as part of the normal day-to-day activity of the diagnostic laboratory, and (ii) a number of culture-negative samples were also positive by immunofluorescence microscopy with MAbs specific for *B. fragilis* polysaccharides (table IX) which, because of the specificity of the MAbs, indicates that *B. fragilis* cells were present in these specimens but were non-viable. Therefore, this

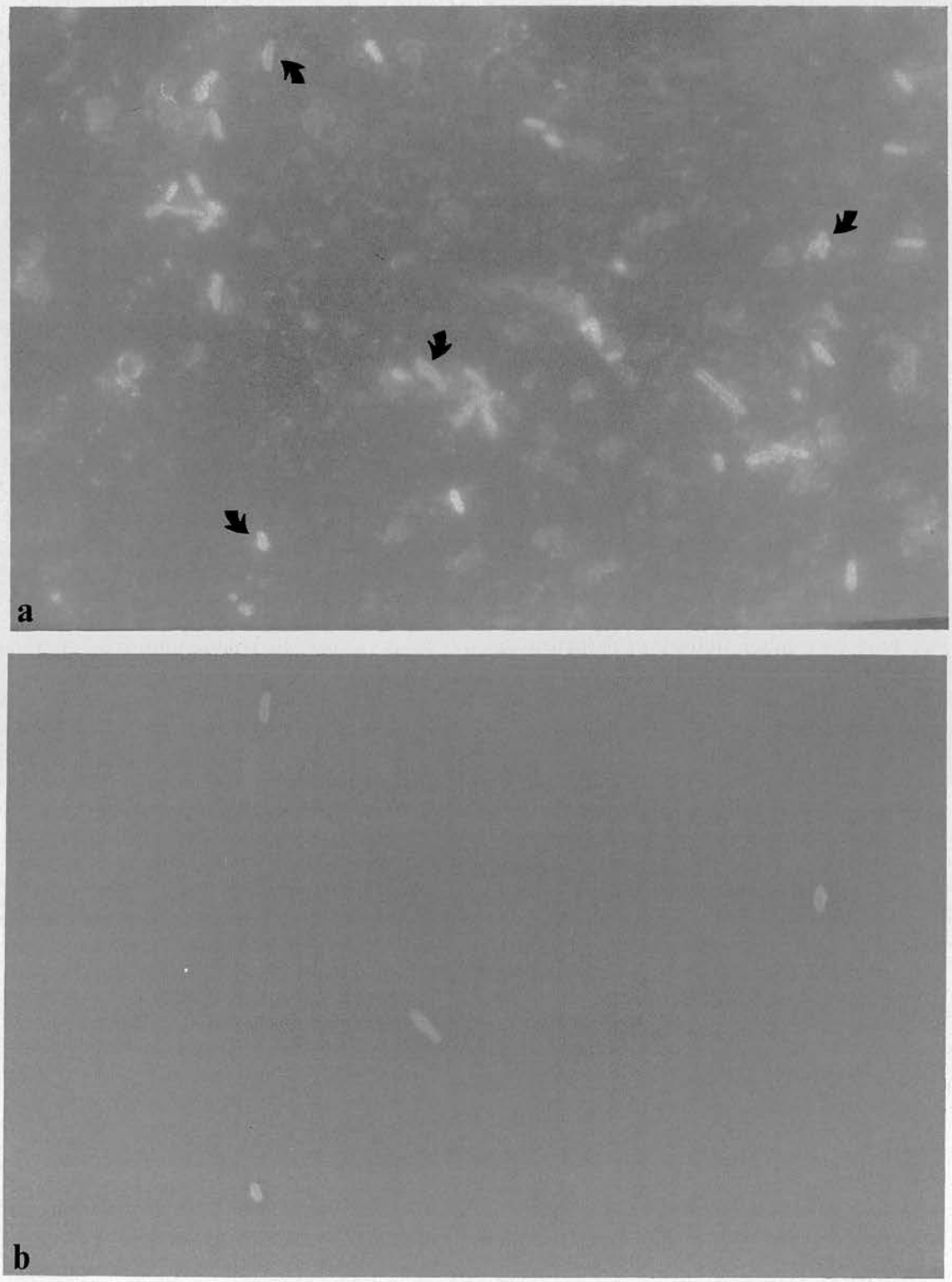

Fig. 3. Micrographs of blood culture labelled with (i) rabbit antiserum to CAg and anti-rabbit immunoglobulin fluorescein conjugate and (ii) mouse MAb QUBf11 and anti-mouse immunoglobulin rhodamine conjugate. The same field of view was photographed with filters to show: **a**, the green fluorescence of the fluorescein; **b**, the red fluorescence of the rhodamine (× 100).

study highlights the problems of detecting obligate anaerobes in clinical specimens by culture alone in a routine diagnostic laboratory and indicates that the CAg is a suitable target for immuno-detection. If the CAg antiserum alone had been used for the detection of *B. fragilis* and related bacteria, two samples that were positive by culture would have been mis-reported as negative. This represents an underestimate of 1–2% of the total of 147 samples examined in the study. In contrast with the current diagnostic laboratory culture methods, the incidence of *B. fragilis* and related bacteria was underestimated by *c.* 14%. Although relatively few blood culture bottles were examined, in all nine instances where *B. fragilis* was isolated, the blood culture was positive with the CAg antiserum. Thus, there is the potential for immediate confirmation

Table IX. Reactivity of pus samples negative for *B. fragilis* and related bacteria, but CAg-positive with MAbs specific for *B. fragilis* NCTC 9343 surface polysaccharides

Sample no.	Reaction with MAb QUBf					
	5	7	8	9	10	11
28	−	+	−	+	−	+
34*	−	+	+	−	−	+
35	−	+	−	−	−	+
57*	−	−	+	+	+	+
68	−	−	+	−	+	+
70*	−	−	+	−	+	+
94	+	−	+	−	+	+

−, negative; +, positive. **B. fragilis* culture positive after re-culture. Four samples were negative with all the MAbs.

of *B. fragilis* in blood culture bottles at the point when growth in the bottle is first observed. The current study examined pus samples and blood culture bottles exclusively. The usefulness of such a test with swab samples remains to be elucidated.

This study also exemplifies the classical polymicrobial infections in which *B. fragilis* are involved and which have been much studied, in particular in relation to *E. coli*.[20] *E. coli* was isolated in association with *B. fragilis* in *c.* 46% of instances; however, *Str. milleri* was present along with *B. fragilis* in 68% of cases in this study. *Str. milleri* is a facultative pathogen that has been isolated from a similar range of body sites to that of *B. fragilis*.[21] The possible synergic interaction between *Str. milleri* and *B. fragilis* warrants further investigation.

Of a total of 45 pus samples from perianal, pilonidal or ischiorectal sites, 39 were positive for *B. fragilis* and related bacteria in this study. The high incidence of *B. fragilis* in abscesses at these sites is interesting in that, although *B. fragilis* is present in quantity in the adherent colonic mucosal flora, it does not predominate in the faecal flora.[3] It is also of interest that in both Study 1 and 2 most of these samples were from male patients (11 male and two female in Study 1; 10 male and three female in Study 2; data not shown). The reasons for this are open to speculation. As all the samples in this study comprised pus that had been drained surgically from an abscess, it is unlikely that the *B. fragilis* detected represents faecal contamination of the specimen. Four of the patients examined in this study had recurrent infections at this site (table VII). Unfortunately, the information in the patients' case histories relating to the antibiotic therapy employed was incomplete, but patient no. 70 was initially reported as culture-negative for *B. fragilis* but positive for *Str. milleri* and is recorded as being treated with co-fluampicil. This sample was positive for the CAg by immunofluorescence and *B. fragilis* was subsequently cultured from the pus when it was re-examined (table IV). This patient had a recurrence of infection at this site 6 months later, from which both *B. fragilis* and *Str. milleri* were cultured.

In conclusion, this study confirms that the immunodominant surface polysaccharides of *B. fragilis* are antigenically variable in both recent clinical isolates and in bacteria present in pus. Thus, these polysaccharides are not suitable targets for the immunodetection of *B. fragilis* in clinical samples. However, CAg is a suitable target and increased the detection of *Bacteroides* and related genera by *c.* 14%.

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MOLECULAR DIAGNOSTICS

Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis

P. D. BROWN, C. GRAVEKAMP‡ D. G. CARRINGTON† H. VAN DE KEMP‡, R. A. HARTSKEERL‡, C. N. EDWARDS*, C. O. R. EVERARD†, W. J. TERPSTRA‡ and P. N. LEVETT§

*University of the West Indies, Faculty of Medical Sciences and * Department of Medicine, Queen Elizabeth Hospital, Bridgetown, Barbados, † Leptospira Laboratory, St Michael, Barbados, West Indies and ‡ Department of Biomedical Research, Royal Tropical Institute, Meibergdreef 39, 1105 AZ, Amsterdam, The Netherlands*

Summary. Early diagnosis of leptospirosis is important because severe leptospiral infection can run a fulminant course. The polymerase chain reaction (PCR) was evaluated for the detection of leptospires in clinical samples from patients with acute leptospiral infection. Blood and urine samples from 71 patients with leptospirosis were examined by PCR, culture or serology. Samples from 44 (62%) patients with the diagnosis of leptospirosis were positive by PCR as compared to 34 (48%) by culture. The presence of leptospires was demonstrated by PCR in 13 patients before the development of antibodies, as well as in two patients who were seronegative during their illness and at autopsy. Samples from 16 patients without leptospirosis were seronegative and culture negative, and also negative by PCR. We conclude that PCR is a rapid, sensitive and specific means of diagnosing leptospiral infection, especially during the first few days of the disease.

Introduction

Leptospirosis is considered to be one of the most widespread zoonoses worldwide.[1] Barbados, the most easterly of all the Caribbean islands, has a population of *c.* 260000 inhabitants and a land mass of 430 km^2. The tropical climate, combined with the predominantly black soils which are slightly alkaline, and the dense population of rodents and dogs distributed throughout the country, make it an ideal environment for the maintenance and spread of leptospirosis.

In Barbados, leptospirosis has been recognised as an important disease affecting man since it was first described in 1939.[2] Since then, the disease has been investigated in hospital patients, dogs, livestock and wild-life, and leptospiral isolates have been identified.[3] Until the end of 1989 only three serovars (*bim*, *copenhageni* and *arborea*), of the 20 or so detected in the Caribbean, were identified among over 100 *Leptospira* isolates from hospital patients in Barbados over the preceding 10-year period. A fourth serovar (*bajan*) has since been identified.[3] Presently, the ratio of presumptive infecting serogroups in hospital patients is Autumnalis 60%, Icterohaemorrhagiae 23%, Ballum 14% and others 3%. On average, 33 cases of severe human leptospirosis are recorded in Barbados each year with an associated case fatality rate of 14·2%.[3]

Accurate diagnosis is important as it gives insight into the extent of the public health problem. Moreover, early diagnosis is particularly important for the clinical management of patients because treatment of leptospirosis can be effective if initiated early. Serology usually does not contribute to early diagnosis of leptospirosis, because antibodies become detectable around the seventh day of the disease. Conventional methods such as dark-field microscopy or culture to detect leptospires in clinical samples are either unreliable or too slow to contribute to a rapid diagnosis.[1,4] Leptospires circulate in the blood of the patient until about the 10th day after the onset of symptoms.

With the introduction of PCR, rapid detection of small numbers of leptospires in clinical samples has become practical due to specific amplification of leptospiral DNA.[5–7] This is important as leptospirosis can run a fulminant course and patients may die before the development of the characteristic clinical manifestations of leptospirosis or the appearance of leptospiral antibodies or both, and, therefore, the disease may go unrecognised. Post-mortem diagnosis may fail because leptospires may die before inoculation of culture medium and specific antibodies may not yet be

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§Correspondence should be sent to Dr P. N. Levett.

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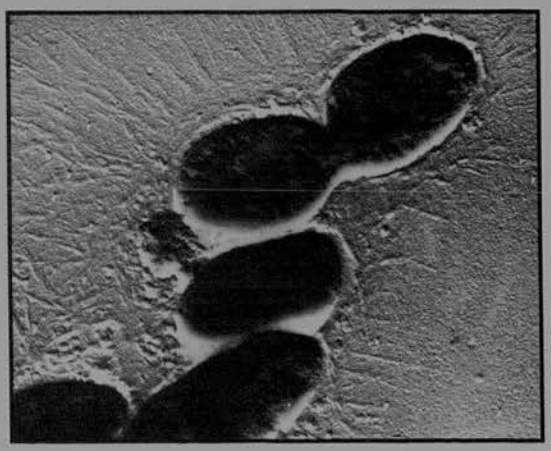

S. Patrick and M. J. Larkin

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S. PATRICK

M. J. LARKIN

The Queen's University of Belfast, Belfast, Northern Ireland, UK

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To our children and in memory of Praful Shirodaria (1939–1994),
Lecturer and colleague at The Queen's University of Belfast

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Preface

This book has evolved from lectures given in Bacterial Pathogenesis, Bacterial Genetics and Immunology courses to Honours year General Microbiology, Medical Microbiology, Genetics and Molecular Biology Students at the Queen's University of Belfast. Our intention is to provide material for the stronger student and hopefully to stretch, rather than confuse, other students. The book should also be of use to students in specialist post-graduate courses as well as post-graduates embarking on research into bacterial virulence. We hope that this text will fill the gap between excellent general texts such as *Mechanisms of Microbial Disease* (Eds Schaechter M., Medoff G., Eisenstein B. I., 2nd Edn, Williams and Wilkins) and more specialist texts such as *Molecular Basis of Bacterial Pathogenesis* (Eds Iglewski B. H., Clark V. L., Academic Press) and *Molecular Biology of Bacterial Infection* (Eds Hormaeche C. E., Penn C. W., Smyth C. J., Cambridge University Press). Our intention is to bring virulence into the context of both the host's immune response and the genetic systems which have evolved in bacteria, by considering molecular interactions. To this end the book covers the host's response to bacterial infection first, before the virulence determinants of bacteria, and ends with the genetic mechanisms which control the expression of bacterial virulence determinants. It is assumed that the underlying infections and pathogenesis of disease caused by individual bacteria are already familiar to the reader, as are fundamental aspects of microbiology, immunology, genetics, biochemistry and cell biology. Although biased towards human pathogens many of the general principles are equally applicable to veterinary pathogens.

1 Pathogenic Bacteria in Context: An Overview

Bacteria are major recyclers on this planet; they degrade organic matter, solubilise inorganic compounds such as clay, contribute to the weathering of rock and degrade toxic pollutants generated by the industrial world. In short, bacteria as a group are capable of a wider range of chemical and metabolic activity than any other group of living organisms. The diversity of bacteria is exemplified by the range of environments that they can inhabit. They can inhabit environments with extremes of temperature, salinity and pressure as well as habitats devoid of oxygen. They have been isolated from environments with temperatures near to freezing and from lakes with both high salinity and temperatures of 44 °C. Thermophilic bacteria grow optimally between 55 and 75 °C; their habitats include naturally occurring hot muds and compost heaps. Some groups of bacteria utilise inorganic compounds as an energy source, some also photosynthesise in the absence of oxygen, while others conduct anaerobic respiration. It could be argued that this is due to the genetic diversity and plasticity of bacteria. Only mankind has matched their ability to survive in such extreme environments, in this case largely due to the evolution of the human brain rather than to the metabolic diversity that is exhibited by bacteria. In all these diverse environments, the resistance of the ecological niche to colonisation (which relates to the degree of adaptation required by the organism) is passive. The environment may be subject to rapid physical and chemical change, but the changes are not brought about by active participation of the environment. The only 'active' competition is from other species competing for the same niche. Bacterial colonisation of an ecological niche inside other living organisms is, however, another matter. Most multicellular organisms have evolved mechanisms to resist colonisation by microbes and this has culminated in the evolutionary splendour of the mammalian immune system; thus in colonising the living host the bacterium is faced with an environment which is actively dedicated to the prevention of its colonisation. Bacteria that can survive within this specialist niche are said to have 'determinants of virulence'; these are the attributes which allow them to survive. Those bacteria which cause abnormalities in the host as a result of colonisation are '**patho**logy **gen**erators' or pathogenic.

A large number of bacteria, the commensal flora, inhabit animals without being pathogenic. All animals have a commensal flora, which in the case of

humans is estimated at 10^{14} microbial cells per person. In fact each human is inhabited by more bacteria than there are humans on the planet. In man, bacteria inhabit the skin, intestine, upper respiratory tract and genito-urinary tract and can be considered technically to be outside the host. The commensal bacteria do not multiply to the detriment of the host, provided they remain in the appropriate ecological niche. Indeed the commensal flora is considered to be largely beneficial to the host and may even be essential for its normal function. Where the commensal bacteria cross the boundaries of these habitats and enter into the host, under what could be considered abnormal conditions (for example as a result of mechanical injury to the host), they may have the potential to become pathogens.

The commensal bacteria and the truly pathogenic bacteria are part of the group of bacteria involved largely in the recycling of organic material and can be nutritionally classified as chemo-organotrophs: organic compounds are their major carbon source and energy is obtained from degradation of organic compounds rather than inorganic compounds or from light. Of this large group of bacteria only a minority are pathogenic and can multiply at the expense of a living host and cause disease. Therefore in the context of the general ecology of the planet and the bacteria which populate it, the pathogenic bacteria could be considered a curiosity; however, in the context of their potential to inflict damage on the human population, they represent a major threat to mankind. It is therefore not surprising that much time and effort has been invested in trying to understand the nature of virulent bacteria, thus enabling the development of means to prevent them from inhabiting this particular ecological niche. With the advent of clean water supplies, safe sewage disposal, successful antimicrobial therapy in the form of antibiotic treatment and vaccination programmes there was a brief period of complacency in the western world when it seemed that bacterial infections were no longer a large threat to the human population. In the context of the whole world, however, the bacterium *Mycobacterium tuberculosis* still kills approximately 3 million people each year, more than any other single infectious disease. Even in the western world bacterial diseases remain a problem. For example in the United States of America sexually transmitted disease caused by *Chlamydia trachomatis* is thought to affect 3 million people annually and gonorrhoea is still the most frequently reported of the officially notifiable diseases, even though AIDS is also officially notifiable.

The study of pathogenic bacteria and an understanding of how they evolve and adapt should lead to better management of disease and more successful targeting of therapy. To do this requires knowledge of a number of areas which relate to both the bacterium and host. The biochemical constituents of the bacterium and the host are the starting point. An understanding of bacterial genetics and the regulation of bacterial gene expression within the host is also essential, as is an understanding of the

interaction of the bacterial constituents with the host cells and systems. The bacteriologist studying virulence has therefore to be an amalgam of biochemist, geneticist, cell biologist and immunologist. With the recent advances in understanding in all these areas, and of the molecules involved in the workings of biological systems in general, bacterial pathogenesis has become an exciting and fast-moving area of study. To date the molecular basis of bacterial virulence is known in part for only a few bacteria and these are by and large the simpler systems where one key characteristic of the bacterium, for example production of a toxin, can be clearly correlated with virulence. For most pathogenic bacteria many different characteristics or factors contribute to virulence and these may vary depending on the stage of infection; virulence is said to be 'multifactorial'. As the pathogenic process cannot be removed from the bacterial interactions with the host, it is very difficult to relate characteristics of the bacterium growing in culture media to virulence in the natural infection. Therefore studies of virulence have two essential requirements: firstly a suitable model system which mimics as closely as possible the natural infection so that putative virulence determinants can be identified; and secondly it is necessary to confirm that putative virulence determinants are expressed by the bacterium during the course of a natural infection. Thus clinical material must also be investigated. One of the most exciting models now being used to identify bacterial genes necessary for virulence which may be expressed **only** in response to host stimuli is in vivo expression technology (IVET). Regions of the bacterial chromosome are isolated, enzymically cut, modified and are inserted back into the genome in such a way that genes which are switched on in vivo can be identified.

As we more clearly understand molecular biology, both in the broad sense of all biological molecules and in the sense of manipulation of deoxyribonucleic acid (DNA), so our understanding of the fascinating co-evolution of pathogenic bacteria and the mammalian immune system will increase. In the long term this should lead to a further reduction of the large impact this minor group of bacteria has on human history.

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A comparison of the haemagglutinating and enzymic activities of *Bacteroides fragilis* whole cells and outer membrane vesicles

Sheila Patrick*, James P. McKenna, Seamus O'Hagan and Evelyn Dermott

Department of Microbiology and Immunobiology, School of Clinical Medicine, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, U.K.

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Patrick, S (Department of Microbiology and Immunobiology, School of Clinical Medicine, Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, U.K.), J. P. McKenna, S. O'Hagan and E. Dermott. A comparison of the haemagglutinating and enzymic activities of *Bacteroides fragilis* whole cells and outer membrane vesicles. *Microbial Pathogenesis* 1996; **20**: 191–202.

The haemagglutinating and enzymic activities of the obligately anaerobic pathogenic bacterium *Bacteroides fragilis* were examined. Outer membrane vesicles are released from the surface of *B. fragilis*. They can be detected by electron microscopy in ultrathin sections and bacterial suspensions after negative staining. Electron microscopy and immunogold labelling with a MAb specific for surface polysaccharide of *B. fragilis* confirmed that the vesicles carried outer membrane associated epitopes. The haemagglutinating activity of whole cells from populations of *B. fragilis* strains NCTC9343, BE3 and LS66 enriched by Percoll density gradient centrifugation for a large capsule (LC), electron dense layer (EDL; non-capsulate by light microscopy) and outer membrane vesicles (OMV) which had been purified by centrifugation from EDL-enriched populations were compared using human and horse erythrocytes. The enzymic activity of OMV, LC- and EDL-enriched populations, as detected by the API ZYM kit, was compared for strains NCTC 9343 and BE3. Purified OMV from the strains examined exhibited both haemagglutinating and enzymatic activity. Haemagglutination by the EDL-enriched population was sensitive to treatment with sodium periodate. The LC-enriched population haemagglutinated only after ultrasonic removal of the capsule. This indicates that the LC masks a haemagglutinin. The results suggest a potential role for OMV in the virulence of *B. fragilis*.

Key words: Bacteroides fragilis; outer membrane vesicles; haemagglutination; extracellular enzymes.

Introduction

The obligately anaerobic bacteria of the genus *Bacteroides* are numerically the predominant component of the normal commensal faecal flora of the colon in all adult humans, where obligate anaerobes outnumber facultatively anaerobic bacteria by about 1000 to 1.[1] *Bacteroides fragilis* is the species most commonly isolated from infections resulting from faecal contamination, despite being outnumbered by other *Bacteroides* species within the faecal flora. It is, however, the predominant *Bacteroides* sp. in the adherent colonic mucosal flora.[2] Although

* Author to whom correspondence should be addressed.

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B. fragilis and related species within the 'fragilis group' of *Bacteroides* spp. (*Bacteroides sensu stricto*) are not members of the normal commensal vaginal flora, they are also responsible for up to half of the upper genital tract, pelvic and uterine infections in women.[3] Infections from which *B. fragilis* has been isolated include abdominal, pelvic, perianal and vaginal abscesses.[4] The majority of *B. fragilis* infections are polymicrobial; for example, *Escherichia coli* and *Streptococcus milleri* are frequently isolated in association with *B. fragilis*.[1,5] The precise virulence determinants of *B. fragilis* remain to be defined; however, surface structures (such as capsular polysaccharides and fimbriae), extracellular enzymes which degrade components of the host tissue and the release of factors which inhibit phagocytic function may all be involved.[6]

Both fimbriae[7,8] and encapsulating structures[9] are implicated in the ability of *B. fragilis* to attach to host cells. Vel *et al.* reported periodate sensitive haemagglutination which did not correlate with the presence or absence of capsules. All of the strains they examined were capsulate by light microscopy but not all haemagglutinated.[10] Pruzzo and colleagues later reported encapsulated haemagglutinating strains in which haemagglutination (HA) was lost after treatment with alpha-glucosidase. It is possible that in these studies the bacterial populations examined contained a mixture of bacteria with different encapsulating surface structures.[7,8]

A major problem in studies of *B. fragilis* is the heterogeneous nature of encapsulating surface structure expression within individual strains.[6] This heterogeneity is obvious when the bacteria are grown in a glucose-rich defined minimal medium such as that of van Tassell and Wilkins.[11] *B. fragilis* may express at least three different types of capsule which can be characterized by electron microscopy of ultrathin sections; namely, a large capsule (LC), a small capsule (SC) and an electron dense layer (EDL). The EDL is not visible by light microscopy. Each of these structures may be produced by different cells within a single strain. Thus to relate a particular surface structure to attachment requires careful definition of the bacterial populations used in the experiment.

LC, SC or EDL populations can be enriched by subculture after Percoll density gradient centrifugation separation.[12] In studies of enriched populations, the EDL population was shown to haemagglutinate human (A, B and O blood groups), horse, guinea pig, rabbit, sheep, mouse and chicken erythrocytes, whereas the LC-enriched population did not.[13] The EDL-enriched population are non-capsulate by light microscopy and fimbriae are not observed by electron microscopy in the strains when they are grown in defined medium broth.[13] It therefore seems that *B. fragilis* is not only capable of producing more than one type of ligand which mediates host cell attachment, but that the expression of these ligands may be subject to within-strain variation.

The release of extracellular enzymes by *B. fragilis* such as hyaluronidase, chondroitin sulphatase, DNAase, protease and lipase, with the potential to degrade components of the host tissues, has long been recognized.[15] Other workers have presented evidence for a link between neuraminidase activity of *B. fragilis* and the ability to agglutinate erythrocytes and attach to tissue culture cells.[16] Macfarlane and colleagues have documented a range of proteolytic and glycosidase activity,[17,18] in studies relating to protease activity of *B. fragilis* as part of the normal intestinal microflora. They reported some protease activity in particulate matter released from *B. fragilis* cells, although 90% of extracellular protease activity was in a soluble form.[17] They suggested that the particulate matter corresponds to vesicles released from the outer membrane. The release

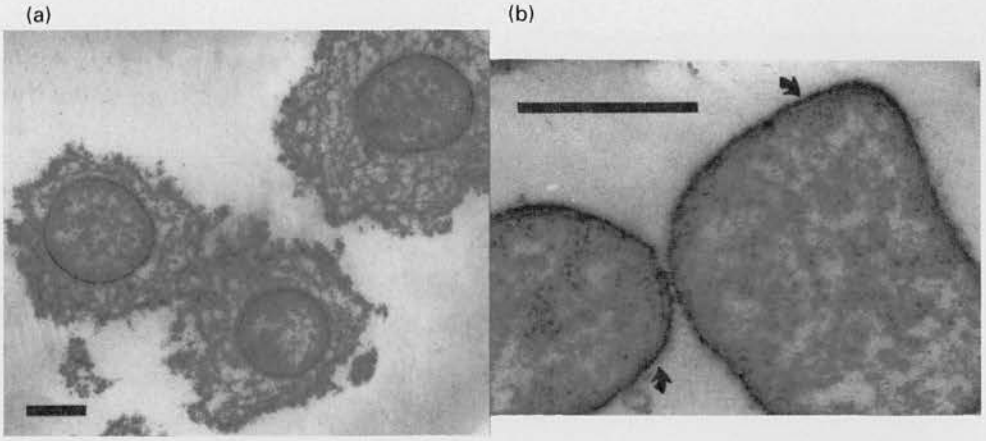

Fig. 1. Micrographs of ultrathin sections of *B. fragilis* strain BE3 enriched for (a) the LC and (b) the EDL by Percoll density gradient centrifugation (bar: 0.5 μm). Note EDL adjacent to the outer membrane (arrow).

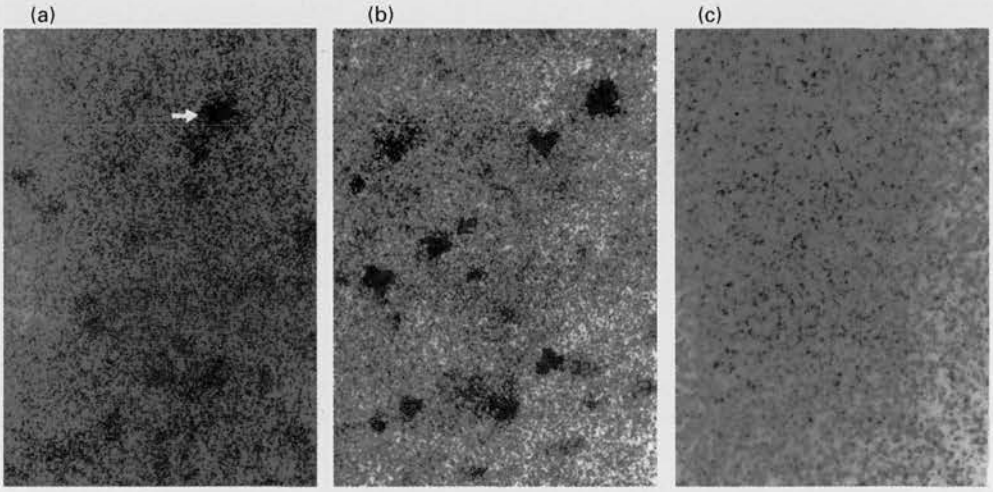

Fig. 2. Slide haemagglutination of human erythrocytes by LC-enriched *B. fragilis* NCTC 9343 after ultrasonication for (a) 30 s, (b) 50 s and (c) 60 s examined by light microscopy. Note that the LC-enriched population only agglutinates erythrocytes after ultrasonication (arrow) and that ultrasonication for 60 s destroys haemagglutinating activity ($\times$ 10 objective).

of outer membrane vesicles (OMV) from a range of Gram-negative bacteria, including *Poryphoromonas (Bacteriodes) gingivalis*, has been reported. It has been postulated that these vesicles are involved in virulence as the reported biological activities of OMV include enzymatic activity, toxicity to host cells and attachment to surfaces, other bacteria and erythrocytes.[19] We have previously reported the observation of OMV in *B. fragilis*.[14]

In the present study we compare haemagglutination and enzymic activity of the EDL-enriched population and preparations of OMV. Enzymic activity was monitored with the API ZYM system which is a rapid and simple method for determining a range of enzymic activities. The sensitivity of the haemagglutinin in the EDL population to sodium periodate treatment was also examined. The

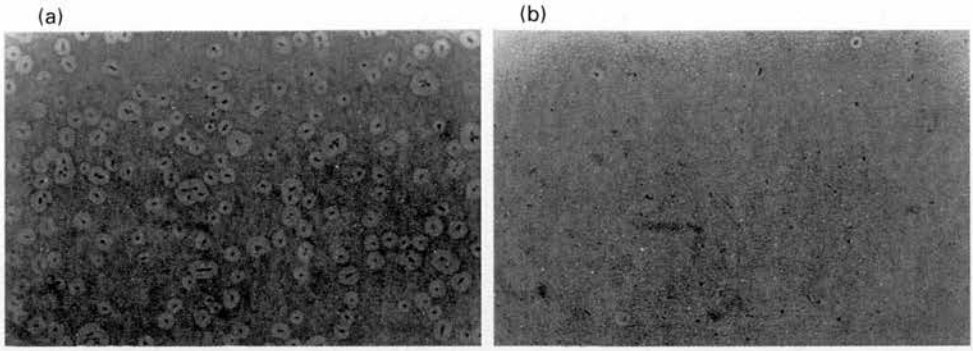

Fig. 3. Eosin/carbol fuchsin stained capsule smear of a *B. fragilis* population enriched for the large capsule (a) before and (b) after ultrasonication for 50 s at an amplitude of 5 μm ($\times$ 100 objective).

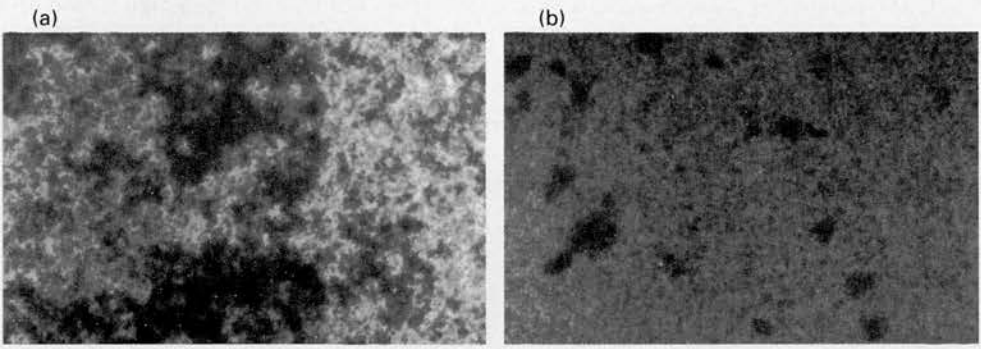

Fig. 4. Slide haemagglutination of human erythrocytes with EDL-enriched *B. fragilis* after incubation of the bacteria for (a) 30 min in PBS and (b) 30 min in 0.01 M sodium periodate. Note erythrocyte agglutination in (a) and reduction in erythrocyte agglutination after treatment of the bacteria with sodium periodate for 30 min in (b). ($\times$ 10 objective).

reactivity of the sodium periodate treated EDL-enriched populations with a number of monoclonal antibodies (MAbs) specific for *B. fragilis* was determined by fluorescence microscopy. The non-haemagglutinating LC-enriched population was tested for HA after ultrasonic destruction of the LC.

Results

Haemagglutinating activity of LC- and EDL-enriched populations after ultrasonic treatment

Populations of strains NCTC 9343, LS66 and BE3, enriched for either the LC, illustrated in Fig. 1(a), or the EDL, illustrated in Fig. 1(b), were ultrasonicated to determine the effect on haemagglutination. Ultrasonic treatment of the EDL-enriched population for longer than 60 s destroyed haemagglutinating activity. The LC-enriched population became haemagglutinating after 30 s ultrasonication, but treatment for more than 50 s destroyed this activity (Fig. 2). Examination of the LC-population by light microscopy after negative staining showed that ultrasonication had destroyed the majority of the large capsules (Fig. 3).

Activities of EDL-enriched populations after sodium periodate treatment

Treatment of the EDL-population with 0.01 M sodium periodate for 30 min reduced haemagglutinating activity (Fig. 4). After 40 min of treatment with 0.01 M sodium

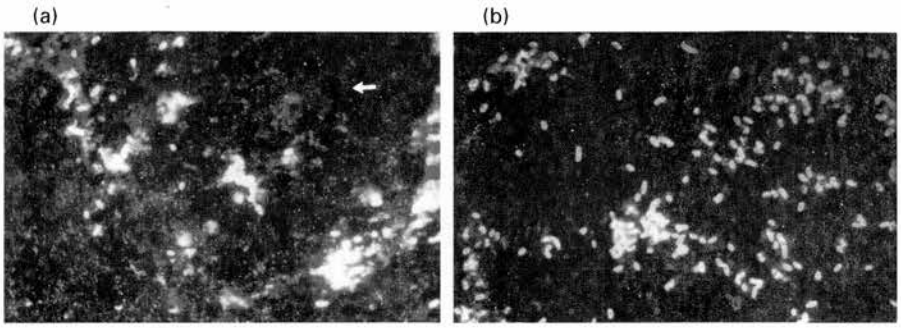

Fig. 5. Micrograph of EDL-enriched population of *B. fragilis* immunolabelled with monoclonal antibody QUBf7 and anti-mouse fluorescein conjugated antibody after incubation in (a) phosphate buffered saline or (b) 0.01 M sodium periodate for 1 h (× 100 objective; micrographs exposed and printed under identical conditions). Note absence of labelled OMV and reduced labelling resulting in clearly defined bacterial shapes after sodium periodate treatment (arrow, labelled OMV).

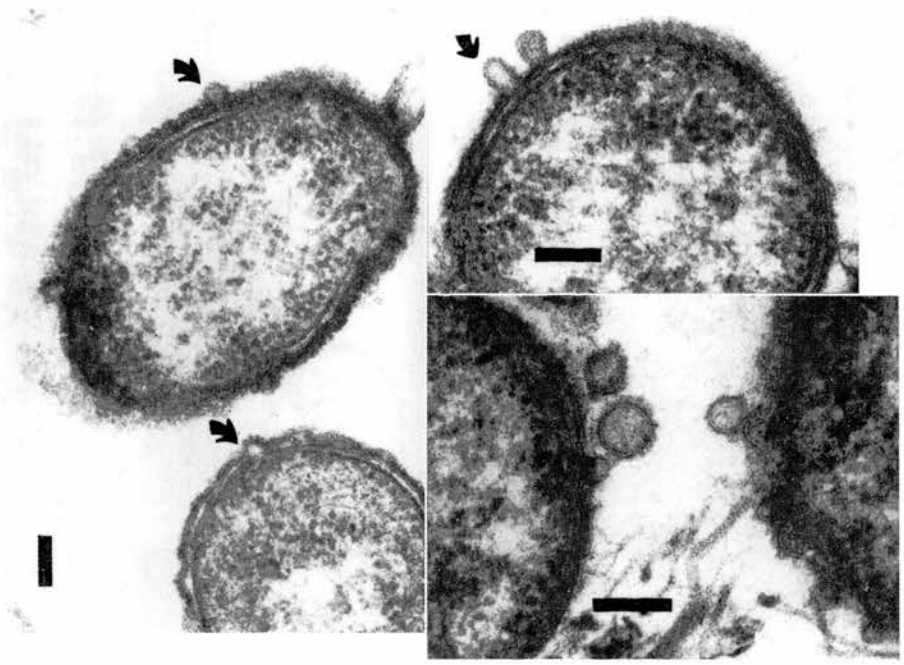

Fig. 6. Examples of OMV release from the surface of *B. fragilis* observed by electron microscopy of ultrathin section (bar: 0.1 μm). Note that the vesicles appear to bud from the outer membrane (arrows) and are bound by a single membrane.

periodate or 30 min with 0.1 M sodium periodate haemagglutinating activity was completely destroyed. The reactivity of the EDL population with MAbs QUBf 5, 6, 7, 9 and 11, specific for high molecular mass surface polysaccharides of *B. fragilis* outer membranes, was reduced but not totally destroyed after treatment for one hour with 0.01 M sodium periodate, although there was a marked reduction in the labelling of bacterial cells and destruction of the OMV labelling (Fig. 5).

Haemagglutinating and enzymatic activity of outer membrane vesicles

The release of single membrane bound vesicles from the surface of *B. fragilis* can be observed by electron microscopy of ultrathin sections (Fig. 6). These are

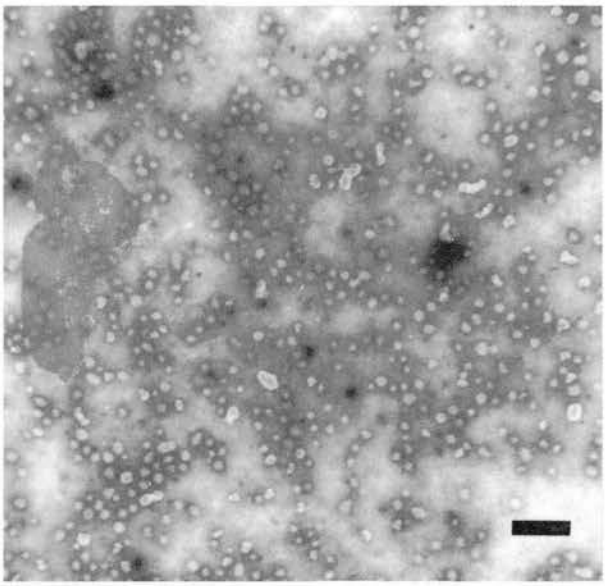

Fig. 7. Electron micrograph of supernatant fraction of EDL-enriched population of *B. fragilis* after centrifugation to pellet bacterial cells and negative staining with methylamine tungstate (bar: 0.5 μm). Note the quantity of outer membrane vesicles.

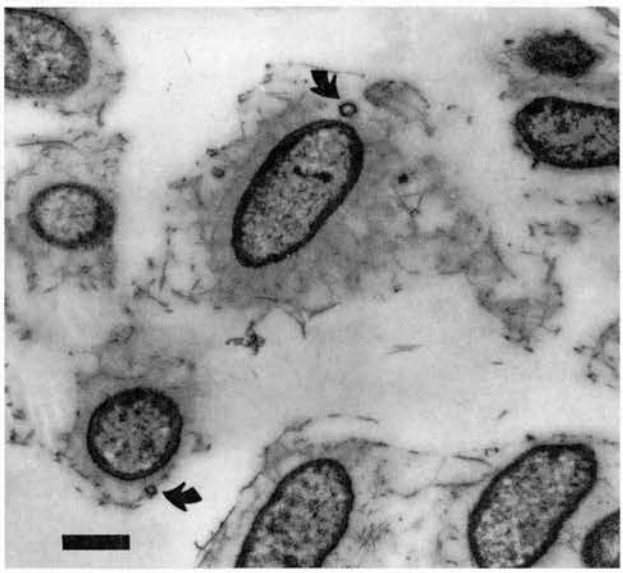

Fig. 8. Electron micrograph of ultrathin section of LC-enriched population of *B fragilis* (bar: 0.5 μm). Note vesicles within the LC (arrows).

released in quantity in broth cultures of the EDL-enriched population and can be observed by electron microscopy after negative staining (Fig. 7). The LC-enriched population also produces OMV, but to a lesser extent, as determined by electron microscopy of negatively stained preparations. Examination of ultrathin sections indicates that some vesicles are apparently trapped within the LC (Fig. 8).

Suspensions of OMV were obtained from EDL-enriched populations of *B. fragilis*

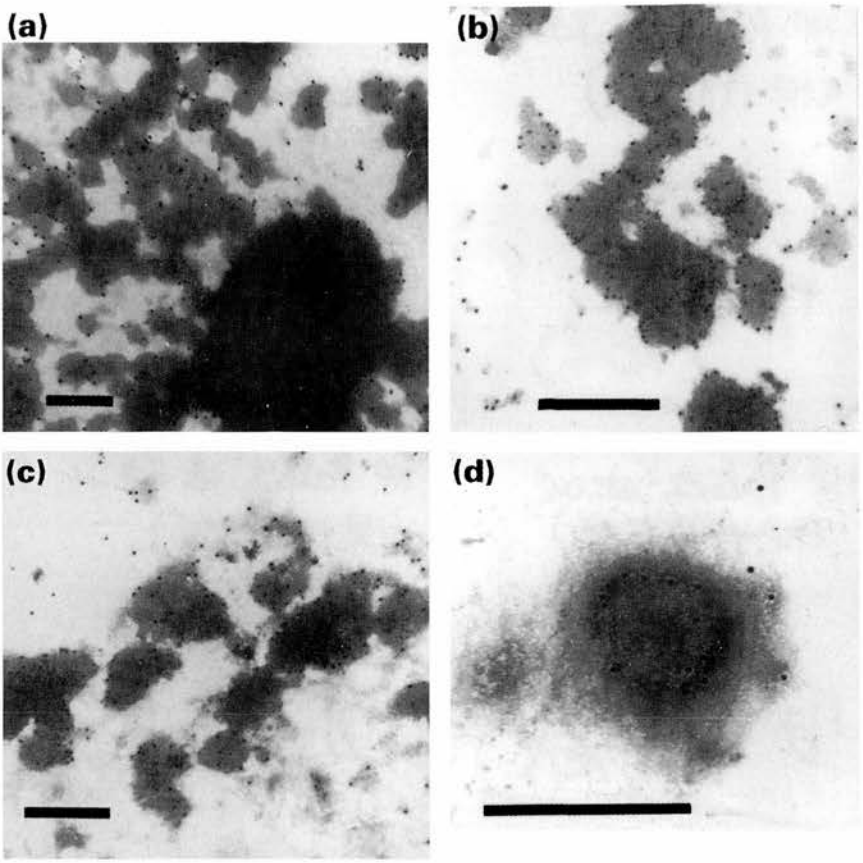

Fig. 9. Electron micrographs of OMV from *B. fragilis* strains (a) NCTC 9343, (b) BE3, (c) LS 66 and (d) single vesicle from NCTC 9343, immunolabelled with monoclonal antibody QUBf5 and anti-mouse gold conjugated antibody (bar: 0.5 μm).

strains NCTC 9343, LS 66 and BE3. Immunogold labelling with MAb QUBf5, specific for a polysaccharide found in outer membrane extracts of *B. fragilis*, was used to verify the presence of bacterial outer membrane associated epitopes (Fig. 9). After polyacrylamide gel electrophoresis and immunoblotting, the antigen labelled by MAb QUBf5 has a ladder-pattern similar to that of the O-antigen of enteric bacteria.[14] All three strains labelled with this MAb, which confirms that the vesicles carry outer membrane associated epitopes and indicates that they originate from the outer membrane by budding as illustrated in Fig. 6. OMV from each of the three strains of *B. fragilis* agglutinated horse and human erythrocytes.

Enzymatic activity of OMV isolated from NCTC 9343 and BE3 EDL-populations (but not strain LS66) was compared with that of whole cells with the API ZYM system. There was no difference in the range of enzymes produced by whole bacteria with either the LC or EDL. The enzymic activities of strains NCTC 9343 and BE3 whole cells and OMV are indicated in Table 1.

Discussion and conclusions

The electron microscopy of ultrathin sections indicates that the vesicles are bound by a single membrane and the MAb labelling results clearly illustrate that OMV

Table 1 A comparison of the enzymic activity of outer membrane vesicles, large capsule- and electron dense layer-enriched whole cells of *B. fragilis* strains NCTC 9343 and BE3

Enzyme	Strain NCTC 9343		Strain BE3	
	Bacterial cells[a]	OMV	Bacterial cells[a]	OMV
Alkaline phosphatase	+	+	+	+
Esterase	+	−	+	−
Esterase lipase	+	+	−	+
Leucine arylamidase	+	−	+	−
Acid phosphatase	+	+	+	+
Phosphohydrolase	+	+	−	+
α-Galactosidase	+	+	+	−
β-Galactosidase	+	+	+	+
β-Glucuronidase	+	−	−	+
α-Glucosidase	+	+	+	+
Glucosaminidase	+	+	+	+
α-Fucosidase	+	−	+	−

Activity of the following enzymes was not detectable with the API ZYM system; lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, β-glucosidase, α-mannosidase.

[a] Large capsule and electron dense layer cells had the same enzyme profile.

carry epitopes associated with the outer membrane of *B. fragilis*. Whether or not there is peptidoglycan present is unknown, although previous reports of similar structures observed in other bacteria indicated that they are lacking in peptidoglycan.[19] The results also indicate a potential role for OMV in the pathogenesis and virulence of *B. fragilis*. The HA and enzymic activity of the OMV suggests that they may be able to attach to host cells and subsequently attack the host cell enzymatically. Enzymic activity associated with OMV is well documented in *Porphyromonas (Bacteroides) gingivalis* which is associated with perdiodontal disease.[21–23] Previous work has documented a range of enzymic activity for *B. fragilis* in relation to the activity of *B. fragilis* in the normal intestinal flora,[17,18] a potential means for the rapid identification of *B. fragilis*[20] and a possible role in virulence.[15] A precise relationship between the production of these enzymes and virulence, however, remains to be determined. There are reports that neuraminidase activity may have a role in attachment to mammalian cheek epithelial cells, the human cell line Intestine 407 and agglutination of erythrocytes of unspecified origin, whereby the neuraminidase exposes a receptor for a *B. fragilis* ligand.[16] In contrast, Namavar *et al.* did not find a correlation between neuraminidase activity and attachment to the colon derived tissue culture cell line WiDr or agglutination of chicken erythrocytes.[24] As the glycosylation of peptides and lipids will vary between different species and different tissue-types within one species, resulting in different glycotypes of the same peptide or lipid,[25] it may be that the *B. fragilis* ligand receptors of the cell-types used in these two studies differ in the degree of sialylation of the oligosaccharides. As the role of oligosaccharide moieties in the regulation of the activities of host cells and molecules becomes more apparent, the role of *B. fragilis* extracellular enzymes in virulence may become clearer. In a study of *B. fragilis* which had been mutagenized in the *nanH* neuraminidase gene, deletion mutants did not grow as well as the wild-type in a rat pouch model of infection. $NanH^-$ insertion mutants, capable of reversion to $NanH^+$, did so after 48 h infection.[26] It will be of interest

to obtain a more detailed picture of the complete enzymic activity associated with OMV and their potential role in virulence.

The ability of OMV to agglutinate erythrocytes may be important, not only in terms of the putative role of OMV in virulence, but also in relation to experimental studies of attachment. In studies of the attachment of *B. fragilis* to host cells, where attachment is monitored by counting of attached bacteria, it is imperative that the quantities of OMV present in suspensions of bacteria are closely monitored. OMV attaching to host cells could block attachment sites and therefore may give false negative results for the potential adhesion of *B. fragilis* to host cells and surfaces.

The results indicate that haemagglutination by the EDL population is mediated by carbohydrate moieties as haemagglutinating activity was destroyed by periodate treatment for 30 min. The epitopes recognized by the high molecular mass polysaccharide specific MAbs were also periodate sensitive; however, although OMV labelling was destroyed, labelling of bacterial cells was of reduced intensity, but not completely destroyed after periodate treatment for 60 min. The effect of sodium periodate treatment on HA by OMV remains to be determined. HA inhibition experiments with hybridoma culture medium containing these MAbs were inconclusive as foetal bovine serum, present in the hybridoma growth medium, inhibited HA by *B. fragilis* EDL-population.[27] Therefore the role of the epitopes recognized by these MAbs in HA remains to be resolved. The LC apparently masks a haemagglutinin as this population haemagglutinated after removal of the LC by ultrasonication. It therefore appears that there is the potential for variation within *B. fragilis* strains from bacteria with a large capsule in which the haemagglutinin is masked to bacteria with the electron dense layer in which the haemagglutinin is exposed.

In conclusion, these results highlight the importance of using bacterial populations which are well defined in terms of not only the types of encapsulating surface structure but also in terms of the presence of OMV in studies of attachment to host cells. Possible roles of OMV in virulence include their ability to disseminate, not only enzymic activity but also possibly toxic activity in the host, both in terms of enterotoxins and endotoxin, whereby the vesicle may penetrate epithelial barriers impermeable to bacterial cells. The association of endotoxic activity with OMV is reported in other species such as *Actinobacillus actinomycetemcomitans.*[28] OMV associated LT-toxin of *Escherichia coli* has also been reported.[29] The release of OMV may prove to have important implications for the pathogenesis of *B. fragilis* as the biological activities of *B. fragilis* LPS appear to be more extensive than early reports in the literature indicate,[30,31] and also in relation to enterotoxigenic *B. fragilis* strains.[32]

The present study raises the possibility that OMV could act as packets of enzymes with the ability to attach to host cells. Whether in so doing they have an impact on the activities of the host cell and/or the nutrition of *B. fragilis in vivo*, and contribute to the pathogenesis of *B. fragilis*, remains to be determined.

Materials and methods

Bacterial strains and culture methods. The strains used were *B. fragilis* NCTC9343 (National Collection of Type Cultures, Colindale Avenue, London, U.K.), LS66 (clinical isolate from an abdominal abscess, Craigavon Area Hospital, Northern Ireland, U.K.)[5] and BE3 (kindly supplied by DM MacLaren, Free University of Amsterdam, NL). Bacteria were grown

in minimal defined medium (MDM) broth which contained, per litre, $(NH_4)_2SO_4$, 2 g; sodium citrate, 0.5 g; vitamin B_{12}, 5 mg; KH_2PO_4, 7 g; K_2HPO_4, 8 g; $MnCl_2.4H_2O$, 10 mg; $MgCl_2.2H_2O$, 20 mg; $FeCl_3.6H_2O$, 0.3 mg; $CaCl_2.2H_2O$, 30 mg; $NaHCO_3$, 4 g; cysteine HCl, 0.5 g; glucose, 10 g; haemin, 5 mg; and resazurin, 1 mg.[11] Cultures were incubated in an atmosphere of H_2 (10%), N_2 (80%), CO_2 (10%) in an anaerobic cabinet (MK III Don Whitley Scientific). Identification was confirmed with the API20A system. Bacterial populations, enriched for either the large capsule (LC) or electron dense layer (EDL; non-capsulate by light microscopy) by Percoll (Pharmacia) discontinuous density-gradient centrifugation[12] were prepared for all three strains.

Electron microscopy of ultrathin sections. Bacterial fixation and staining were carried out as previously described.[33] Briefly, preparations were fixed in glutaraldehyde (2.5% v/v) in cacodylate buffer (0.1 M; pH 6.8) for 1 h at 4°C in the dark, followed by osmium tetroxide (1% w/v) in cacodylate buffer for 3 h at room temperature in the dark. Ruthenium red stain (1 mg/ml) was included at both fixation stages. After dehydration in a graded series of alcohols the preparations were embedded in Spurr or LR white resin.

Preparation of outer membrane vesicles. Late exponential-phase EDL-enriched *B. fragilis* strains NCTC 9343, BE3 and LS66 cultures in MDM broth (20 ml) was centrifuged at $7245 \times \boldsymbol{g}$ in an MSE High Speed 18 Centrifuge to pellet the bacterial cells. The supernatant fraction was drawn off and washed twice by centrifugation at $30790 \times \boldsymbol{g}$ in a Beckman L5-50B ultracentrifuge. The supernatant fraction was removed and the pellet was resuspended in 1 ml of either phosphate buffered saline (0.01 M), pH 7.4 (PBS) or 0.15 M NaCl (saline). The presence of OMV and the absence of bacteria was confirmed by light microscopy and electron microscopy. Preparations for electron microscopy were either negative-stained directly using methylamine tungstate 2% w/v in distilled water or after immunogold labelling. Suspensions in distilled water were applied to Formvar/carbon coated nickel grids. For immunolabelling, the grids were then placed sequentially in (i) bovine serum albumin (BSA; 1% w/v) in PBS, for 15 min, (ii) hybridoma culture supernatant for 2 h, (iii) BSA (0.1% w/v) in 20 mM Tris-HCl saline, pH 8.2, (TBS) prior to washing in a stream of drops of TBS (total volume approximately 8 ml), (iv) BSA-TBS (1% w/v) for 15 min, (v) anti-mouse gold conjugated antibody (Janssen) for 2 h, (vi) washed as in (iii) in BSA-TBS (0.1% w/v) followed by distilled water. The grids were observed with a Phillips CM10 electron microscope. All incubations were carried out at room temperature.[12,14,34]

Ultrasonic treatment. Bacterial suspension (1 ml) of approximately 3×10^9 cfu/ml in PBS was sonicated in an MSE 150 soniprep for various time periods at an amplitude of 5 μm in an Eppendorf 1.5 ml plastic centrifuge tube. After ultrasonic treatment the sample was centrifuged at $7245 \times \boldsymbol{g}$, the supernatant fraction removed and the pellet resuspended in PBS. The bacteria were negatively stained with eosin/carbol fuchsin. A loopfull of bacterial culture was applied to a clean dry slide and mixed with a loopfull of carbol fuchsin (Ziel-Nielsen's carbol fuchsin diluted 1:5) and allowed to stand for 30 s. This was then mixed with a drop of eosin staining solution (four parts water-soluble yellowish or bluish erythrosin 10% w/v in distilled water to one part rabbit serum which has been heated at 56°C for 30 min) and allowed to stand for 1 min. The suspension was then spread along the slide with a second clean slide, allowed to dry and examined by light microscopy for the presence or absence of capsules and whole bacterial cells.[12]

Sodium periodate treatment. Sodium periodate (0.1 and 0.01 M in PBS) was prepared immediately prior to use. Bacterial suspensions (approximately 2×10^9 cfu/ml) were washed twice by centrifugation, resuspended in sodium periodate and incubated in the dark at 37°C for different time periods. Suspensions were then washed twice by centrifugation to remove any residual sodium periodate. The absence of sodium periodate activity was monitored by wetting starch/potassium iodide impregnated paper (Johnsons of Hendon Ltd) with the supernatant fraction. The starch paper turns blue with sodium periodate activity. Bacterial suspensions which were incubated in PBS without sodium periodate were processed in parallel as a control.

Haemagglutination. Heparinized whole human blood group O and horse blood erythrocytes were washed three times by centrifugation at $200 \times \boldsymbol{g}$ and resuspended to 2% packed cell volume. Bacterial (approximately 2×10^9 cfu/ml) or OMV (prepared as above)

suspensions (20 μl) in PBS were mixed with the erythrocyte suspension (20 μl) on a chilled ceramic tile and agitated by hand. A positive result was recorded if haemagglutination occurred within 5 min. Suspensions were also examined for haemagglutination with an inverted light microscope.

Use of API ZYM kits to assess enzymatic activity. Bacterial suspensions of approximately 1×10^9 cfu/ml or OMV, prepared as above, in saline (40 μl) were dispensed in the cupules of the API ZYM kit. The kits were incubated for 4 h at 37°C and reagents added according to the manufacturer's instructions. Enzymic activity was recorded as positive if a score of 1 or greater was obtained after assessment of the colour intensity using the manufacturer's colour chart as a guide.

Immunofluorescence microscopy. Bacterial suspensions in PBS were applied to multiwell microscope slides, dried at 37°C and fixed in methanol at −20°C for 10 min. The bacteria were then reacted with mouse monoclonal antibodies followed by anti-mouse immunoglobulin G (heavy and light chain) conjugated to fluorescein isothiocyanate (FITC) as previously described.[12] Slides were examined with a Leitz fluorescence microscope.

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Infection associated with medical devices

M. M. Tunney[1,2], S. P. Gorman[1] and S. Patrick[2]

[1]*School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK*
[2]*Department of Microbiology and Immunobiology, School of Clinical Medicine, Royal Victoria Hospital Site, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK*

The use of temporary or permanent implants fabricated from polymeric biomaterials within the body has increased dramatically in recent years. Unfortunately, bacteria adhere to the surface of these medical devices, producing biofilms. Device-related infection may result in tissue destruction, systemic dissemination of the pathogen and dysfunction of the device, causing increased morbidity and mortality. These infections are resistant to immune defence mechanisms and are difficult to treat with antimicrobial agents. Removal of the device may be necessary with attendant distress to the patient and cost. Considerable research effort is currently directed towards reducing, if not eliminating, infection of medical devices. Strategies under investigation include the use of electric fields to improve antibiotic therapy, physiochemical modification of the biomaterial surface to create anti-adhesive surfaces to prevent bacterial adhesion and incorporation of antimicrobial agents into medical device polymers.

Keywords: Medical devices; prostheses; infection; biofilm; adhesion; antibiotic resistance.

INTRODUCTION

The development of synthetic biomaterials for temporary or permanent implantation constitutes a significant advance in modern medicine. The increased use of medical devices such as intravenous catheters, endotracheal tubes, ureteral stents, prosthetic heart valves and orthopaedic devices led Gristina to predict that 'Ultimately, almost every human in technologically advanced societies will host a biomaterial' [1]. Unfortunately, infection which can result in considerable morbidity and mortality in patients is a frequent complication associated with the use of these devices (Table 1). Infections associated with medical devices are typically caused by bacteria of the normal microbiota, such as those of the skin, and are characterized by a low inoculum and poor response to antibiotic therapy. We now review the factors which affect bacterial adhesion to medical devices and the strategies currently being employed in an attempt to improve treatment of such infections.

MICROBIAL ADHESION

Microorganisms have a natural tendency to adhere to surfaces as a survival mechanism. This can occur in any environment, not just the living host but also, for example, in natural aquatic environments and industrial systems. The general outcome of bacterial colonization of surfaces is the formation of an adherent layer, or biofilm, composed of bacteria embedded in an organic matrix. The organic matrix is usually generated by the bacteria, although within the environment of the living host it is possible that material derived from the host will also be present.

It has been suggested that bacterial adhesion to a surface occurs in a sequential manner, being

Address for correspondence: Michael Tunney, Department of Microbiology and Immunobiology, School of Clinical Medicine, Royal Victoria Hospital Site, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK.

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Table 1. Infection rates associated with implanted medical devices

Medical device	Incidence of infection (%)	Typical pathogens
Orthopaedic prostheses	1–2	*S. aureus, S. epidermidis, E. coli, Propionibacterium acnes, Bacteroides fragilis*
Prosthetic heart valves	0.4–2	*S. aureus, S. epidermidis*
Endotracheal tubes	9–70	*Ps. aeruginosa, S. aureus, E. faecalis, Candida* spp
CAPD catheters	70–100	*S. epidermidis, Ps. aeruginosa*
Urinary catheters/stents	15–20	*Enterococcus* spp. *S. aureus, E. coli*
CSF shunts	1.5–6	*S. epidermidis, S. aureus*

influenced by both physical and chemical factors [2]. These may include the properties of the material surface as well as the nature of the molecules adsorbed onto the surface from the surrounding fluids to form a layer termed the conditioning film. Although no irrefutable theory has been developed to explain the fundamental mechanisms of bacterial adhesion, most hypotheses recognize four phases in the process (Fig. 1).

Microorganisms may arrive at the surface of a medical device via a number of different routes: introduction during the insertion of a medical device, transient bacteraemia, or through the site of exit of the medical device from the body. Attachment phase 1 involves movement of the bacteria to the device surface by either Brownian motion or bacterial chemotaxis and motility.

This is followed by phase 2, requiring initial adhesion according to the DLVO (Derjaguin and Landau, Verwey and Overbeek) theory. At a distance of approximately 50 nm the bacterium will be attracted to the surface by van der Waals' forces. Frequently, both the bacterium and device polymer possess the same charge and, therefore, the electrostatic repulsive force becomes higher the closer the bacterium moves to the surface. These forces, acting in concert, govern the long-range interactions between the bacterium and the device surface. Closer to the surface are two regions where the attractive forces are greater than the repulsive forces. These are called the secondary minimum, which occurs at approximately 10 nm from the surface, and the primary minimum, at approximately 1 nm from the surface. These are separated by a maximum energy barrier where repulsive forces predominate. Bacterial adhesion at the secondary minimum is reversible, and bacteria may become detached as a result of fluid shear forces. Bacterial surface structures, such as fimbriae, flagella or surface polysaccharides, are thought to play a key role in the ability of some bacteria to overcome this maximum energy barrier and thus allow the bacteria to reach the primary minimum [2]. These bacterial structures are sometimes termed 'bridging polymers'. When the bacterium reaches the primary minimum, adhesion becomes irreversible.

At phase 3, specific interactions, involving bonding (covalent, ionic and hydrogen), occur between the surface and the bacterium.

In phase 4, colonization occurs, with the attached bacteria synthesizing and secreting a variety of exopolymeric substances of which extracellular polysaccharides (EPS) account for approximately 90% of the mass. The bacteria become encased in this material, which is sometimes referred to as the glycocalyx. Further bacterial cell division produces microcolonies within the matrix. Under suitable conditions, a continuous biofilm rapidly becomes established (Fig. 2). Extensive biofilm has been identified on endotracheal tubes retrieved from patients in intensive care after only 24 hours intubation [3]. The biofilm may subsequently be enlarged by the attachment of more free-living bacteria from the surrounding fluid medium along with bacterial replication and exopolysaccharide production.

The clinical impact of microbial adhesion to biomaterials is that a formed biofilm may result in tissue destruction, dysfunction of the prosthetic device and systemic dissemination of the pathogen. Within the biofilm, the bacteria may be protected from the host defences and the action of antimicrobial agents. These infections are therefore difficult to treat and this may

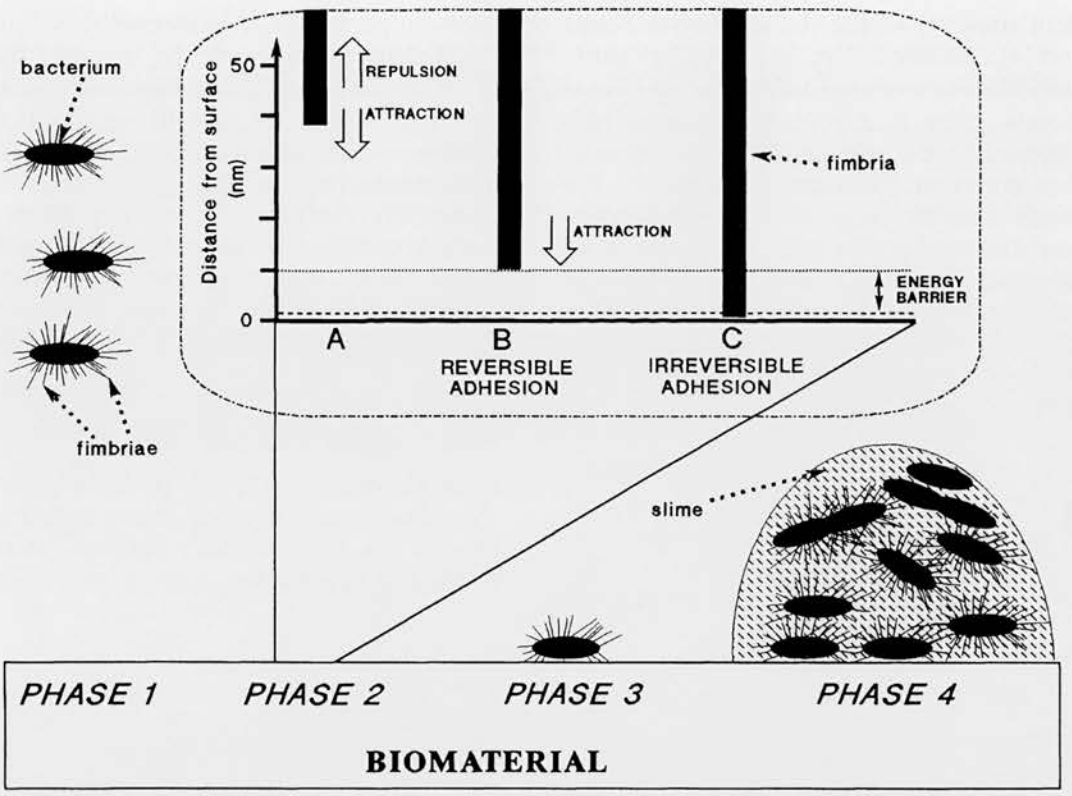

Fig. 1. Schematic diagram of bacterial adhesion to a biomaterial surface. Phase 1: transport of bacteria to the biomaterial surface. Phase 2 (boxed inset): A, attractive van der Waals forces overcome repulsive electrostatic forces and the bacteria move closer to the surface; B, bacteria at secondary minimum (dotted line) adhere reversibly to biomaterial surface; C, bacteria use fimbriae (or bridging polymers) to overcome the energy barrier and reach the primary minimum (dashed line) where adhesion is irreversible. Phase 3: bonding of bacteria to surface. Phase 4: colonization of surface and formation of a bacterial biofilm.

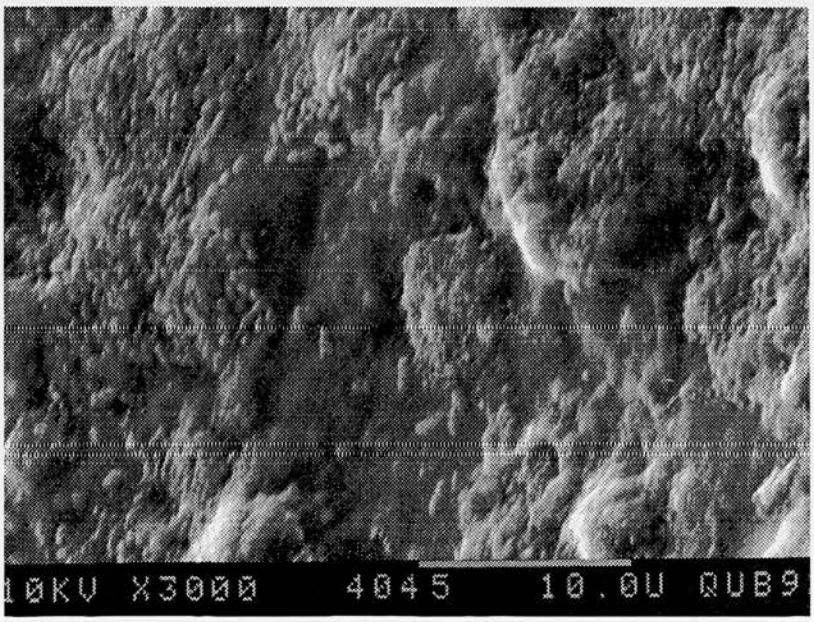

Fig. 2. Scanning electron micrograph of a bacterial biofilm on a retrieved ureteral stent.

necessitate removal of the device. Gristina and Costerton [4] described that, in 19 of 25 patients with medical device-related infection, the causative bacteria grew in glycocalyx-enclosed biofilms adherent to the surface of the biomaterial. The most common pathogens isolated in the study were *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. They concluded that microbial adherence and biofilm growth were fundamental to infection involving biomaterials, and that it may be a factor in the susceptibility to infection of patients with implanted devices.

Bacteria have been isolated from within biofilms on the surfaces of a wide variety of medical devices, including urinary catheters/stents, continuous ambulatory peritoneal dialysis (CAPD) catheters, endotracheal tubes and orthopaedic prostheses [5]. The bacteria most commonly isolated from within these biofilms are *S. aureus*, *S. epidermidis*, *E. coli* and *Ps. aeruginosa* (Table 1).

Several studies have recently examined biofilm formation on ureteral stents. Reid et al [6] found that of 30 retrieved ureteral stents, 90% had adherent pathogens on the surface, of which 45% were present in low numbers (10^1–10^2 adherent organisms cm^{-3} of suspending buffer), and 55% were present in small and large microcolony biofilms ($>2\times10^2$–10^7 adherent organisms cm^{-3}). The organisms isolated were mainly Gram-positive cocci with a smaller number of Gram-negative rods. While no association was found between the presence of bacterial biofilm and the development of symptomatic infection, the adherent organisms were isolated despite prophylactic antimicrobial therapy being given to all the patients. In another study of 40 retrieved ureteral stents, a profuse biofilm ($>10^4$ cfu cm^{-2} of stent surface) was present on 28% of stents [7]. Gram-positive cocci accounted for seven of the 13 organisms isolated; *Enterococcus* spp for five of the isolates and *S. aureus* for two. Gram-negative rods isolated included *E. coli* (two isolates) and *Ps. aeruginosa* (one isolate). The ureteral stents in this study were also colonized by bacteria despite the use of a peroperative antibiotic in every patient at stent insertion. Gorman et al [8] reported that a viable microbial biofilm was present on 25 of 32 Tenchkoff catheters retrieved from CAPD patients. The most commonly isolated bacteria in this study were coagulase-negative staphylococci (in particular, *S. epidermidis*), *S. aureus* and *Ps. aeruginosa*. Fitzgerald [9] reported that 1–5% of indwelling hip prostheses became infected, whereas Nasser et al [10] reported that the incidence of infection is approximately 1% over the lifetime of the hip prosthesis. The most commonly isolated bacteria in these studies were *S. aureus*, *S. epidermidis* and *Ps. aeruginosa*. It is likely, however, that the incidence of obligately anaerobic bacteria, particularly in prosthetic-joint infection is underestimated [11].

Factors which influence adhesion

It is generally accepted that three important components are involved in the initial adhesion process to biomaterials: bacteria, suspending fluids and substrata.

Bacteria

Bacterial cell-surface components, such as fimbriae and polysaccharides, are considered to promote adhesion to surfaces. Filamentous structures extending from the bacterial cell surface, for example fimbriae or polysaccharides, may help to initiate attachment by bridging the electrostatic repulsive forces at the surface of the device [2], as discussed above. Exopolysaccharide production may therefore be critical to biofilm formation during both the period of initial interaction of the bacterium with the surface to be colonized and during the later stages of biofilm formation. Clearly, an understanding of the nature and control of expression of these molecules is central to our understanding of the factors which influence biofilm formation. In particular, it is clear that not only are bacterial polysaccharides complex in terms of their structure and components, their production is variable both between and within strains [12, 13] and may be subject to both phenotypic and genotypic variation. It is likely that different polysaccharides produced by an individual strain of bacteria may be involved at different stages of the attachment process. This topic is reviewed in depth by Allison [14].

Specific interactions between bacterial surface molecules and host cell surfaces are well documented [15] and it is likely that similar interactions occur between bacteria and host components which may coat the surface of an implanted device (see below).

Suspending fluids

On implantation in the body, medical devices are usually surrounded by suspending fluids such as urine, blood, saliva and synovial fluid. These body fluids contain various proteins such as fibronectin, laminin, fibrin, collagen and immunoglobulins which are almost immediately coated onto the biomaterial surface to form the conditioning film [16]. Bacteria may possess binding sites for glycoproteins and proteoglycan within the conditioning film, thereby promoting their adhesion (see above). Whether or not the biomaterial is colonized by bacteria or host cells may depend on which first contacts the biomaterial, or which one is a better competitor for the surface. Cheung and Fischetti [17] showed that fibrinogen or fibrin is the dominant plasma mediator of staphylococcal adherence to intravascular catheters. Gorman et al [18] showed that the formation of a conditioning film on polyurethane and silicone peritoneal catheters by pre-treatment with artificial spent dialysate significantly reduced bacterial adherence to their surfaces.

The conditioning films formed on ureteral stents have been recently described [19]. An examination of the surface of retrieved stents by X-ray photoelectron spectroscopy revealed the presence of adsorbed nitrogen, and in some cases calcium and phosphate. Energy dispersive X-ray analysis confirmed the presence of calcium, magnesium, phosphorus and carbonate apatite crystals. The changes in surface composition were not thought to be due to leaching or degradation as scanning electron microscopy (SEM) showed deposits on the surface of the material. The authors also described an in-vitro study in which a stent incubated in urine for 24 hours had an adsorbed conditioning film, making its surface quite different from a control stent which had not been incubated in urine.

The variable composition of the suspending fluid is also thought to have an impact on bacterial adhesion. For example, within the urinary tract, electrolytes, pH, urea and creatinine concentrations have all been shown to affect the adhesion process. Reid et al [20] showed that an environmental pH in the range 5–8 and a low urea concentration increased adherence. In a similar study [21], it was shown that adhesion of *S. epidermidis* was significantly increased in the presence of magnesium and calcium, but significantly decreased in the presence of the chelating agent ethylenediaminetetraacetic acid. Altered in-vitro adherence of coagulase-negative staphylococci to surfaces has been demonstrated if the organisms are grown in an environment equilibrated with 5% CO_2 [22]. Similarly, Bonner et al [23] described a significant increase in the adherence of a ureteral stent biofilm isolate, *Enterococcus faecalis*, to polyurethane when it was grown in an atmosphere equilibrated with 5% CO_2.

Substrata

As with the bacterial and fluid components, there are certain properties of a biomaterial that can influence adhesion and biofilm formation [24].

Although a biomaterial surface may appear smooth to the naked eye, SEM invariably shows it to be uneven and possessing irregularities. These defects have often been thought to provide niches for bacterial adhesion. Reid et al [25] showed by SEM that the surface of urinary catheters was uneven. Similarly, Gorman et al [26] showed by confocal laser scanning microscopy that the surface roughness or microrugosity of peritoneal catheters increased during implantation, and that adherence of *S. epidermidis* to these rough catheters was significantly greater than to unused (control) catheters. Factors which may be involved in the production of increased roughness during medical device implantation may include shear forces arising from fluid dynamics and host or bacterial degradation of the device biomaterial. In both studies, the authors suggested that surface irregularities could offer attachment points for infecting bacteria. Bacterial adherence to materials of different surface microrugosity has been investigated. An experimental polymer (Vivathane) which had an extremely smooth surface, was shown to prevent bacterial adherence and biofilm formation when used as a biliary stent [27]. In contrast, C-Flex and polyethylene stents which were shown to have surface irregularities were colonized easily by bacteria, and the lumen became blocked after 6 and 10 days, respectively.

The influence of bacterial and biomaterial surface hydrophobicity on microbial attachment remains unclear. Illustrating this problem, a hydrophilic *E. coli* isolate and a hydrophobic *S. aureus* isolate

showed no difference in adherence capability to either hydrophobic latex or hydrophilic polymer-coated urinary catheters [28]. In contrast to the results of this study, Roberts et al [29] showed that hydrophobic bacteria did not adhere to a hydrophilic catheter surface, however, Reid et al [30] reported that adherence of *Lactobacillus acidophilus* to polymer surfaces increased with increasing hydrophobicity of the surface, but in further work, Reid et al [31] reported that a conditioning film is rapidly deposited on a biomaterial on insertion in the body, altering its surface properties. They therefore suggested that it was misguided to assume that surface hydrophobicity measurement prior to device insertion was relevant to bacterial adhesion in vivo.

ROLE OF THE BACTERIAL GLYCOCALYX

Apart from its adherent properties, which have been described previously, a number of roles have been suggested for the bacterial glycocalyx. These include resistance to antibiotic therapy, attenuation of the host response and retention of host nutrients.

Treatment of biomaterial-associated infection is extremely difficult because, although the infection may be temporarily controlled with large doses of antibiotics, it quickly recurs on withdrawal of therapy. This problem was demonstrated in early work by Nickel et al [32] who reported tobramycin resistance of *Ps. aeruginosa* grown on Silkolatex catheter material. Free-living cells of *Ps. aeruginosa* were shown to be completely killed by 50 μg ml^{-1} of tobramycin. Discs of catheter material were then exposed to the flow of artificial urine containing cells of *Ps. aeruginosa*. A thick adherent biofilm formed on the surface of the discs after 8 hours, and a significant number of cells within the biofilm were found to be still viable after 12 hours of exposure to 1000 μg ml^{-1} of tobramycin. After dispersal of the biofilm into a culture medium, the cells were again killed by 50 μg ml^{-1} of tobramycin. The results of this study suggested that the organism did not develop resistance to tobramycin as a results of genotypic changes but rather that growth within the biofilm conferred a level of resistance to antibiotics. The exact mechanism for this antimicrobial resistance remains controversial. Initially, it was thought that the glycocalyx could act as a simple diffusion barrier to inhibit the access of antibiotic molecules to the bacterial cell [33]. Alternatively, it was suggested that the negatively charged glycocalyx could bind positively charged antibiotic molecules, such as those of the aminoglycoside group [34]. However, this could not account for the resistance to neutral and negatively charged antibiotics. More recently, Nichols [35] suggested that antimicrobial resistance was not due to low permeability but due to the production of extracellular enzymes such as β-lactamase, which can inactivate antibiotics diffusing into the biofilm, thereby reducing the concentrations reaching the bacteria positioned closer to the material surface. Adding support to this theory, Giwercman et al [36] demonstrated that *Ps. aeruginosa* growing in a biofilm adherent to catheter material can produce 32 times more β-lactamase than the same cells in suspension, and therefore has a greater potential to inactivate antibiotics than do the suspended microorganisms. Compounding this problem of biofilm resistance, Naylor et al [37] and Costerton et al [38] have both suggested that antibiotic resistance is fundamentally a feature of phenotypic alterations of bacterial metabolism which occurs as a result of bacterial growth within a biofilm.

Production of a bacterial glycocalyx may also act to impede the host immune system. Peters et al [39] showed that the production of glycocalyx by *S. epidermidis* interfered with the normal phagocytic function of polymorphonuclear leukocytes, decreased production of T and B cells and inhibited immunoglobulin production.

Other work has shown that the glycocalyx can optimize growth conditions by retention of nutrients such as iron close to cell surfaces and it may also act as a barrier against iron sequestering proteins of the host such as lactoferrin and transferrin [40].

PREVENTION AND TREATMENT OF BIOMATERIAL-RELATED INFECTION

Given the persistent nature of biomaterial-centred infections and the possible severe consequences for the individual patient, measures to prevent such infections are urgently needed [41]. Long-term oral antibiotic therapy has not been shown to be effective in preventing

such infections, and, indeed, has been shown to have a detrimental effect on the normal flora with development of resistant organisms. Strategies which have evolved for the treatment of biomaterial-related infections include improved antibiotic therapy, physicochemical modification of the biomaterial surface to create anti-adhesive and hence anti-infective surfaces, and coupling or incorporation of antimicrobial agents to or into polymers to prevent bacterial colonization.

Improved antibiotic therapy

Growth of bacteria within a biofilm on the surface of a medical device confers a level of resistance to antibiotics, which may render conventional antibiotic therapy ineffective [32]. Several studies have described recent developments which have attempted to overcome this problem and improve antibiotic efficacy.

The inherent resistance of biofilm bacteria to antibiotic therapy can be overcome when the antibiotic is used in the presence of a relatively weak electrical field. Recent work in this novel area has described how the presence of an electrical field lowered the concentrations of β-lactams, amines and macrolide antibiotics required to kill biofilm bacteria [42]. The authors suggested that the weak electrical field may drive the antibiotic through the biofilm matrix. In a related study, Davis et al [43] reported that iontophoresis (compound delivery by charge) with gold, carbon and platinum electrodes effectively reduced or eliminated both Gram-positive and Gram-negative inocula in synthetic urine.

Another strategy which has been employed to improve antimicrobial activity is the use of a surface-active agent in association with an antibiotic. In a recent study, Soboh et al [44] described the use of a polycationic protein, protamine sulphate, in combination with the fluoroquinolone, ciprofloxacin, against a clinical isolate of *Ps. aeruginosa*. Protamine sulphate (50 μg ml^{-1}) combined with ciprofloxacin (0.5 μg ml^{-1}) reduced the number of viable cells by 99%, whereas protamine sulphate or ciprofloxacin alone resulted in an 108% increase and 59% decrease respectively, after 24 hours. The observed synergistic effect was considered to be due to protamine sulphate denaturing the glycocalyx, thereby enhancing ciprofloxacin penetration through the biofilm or, alternatively, due to an alteration in membrane permeability and a dilation of ionic channels by protamine sulphate which facilitated transport of ciprofloxacin to the cytoplasm.

Physicochemical modification of the biomaterial surface

Surface modification of a biomaterial may lead to altered surface properties and thus to altered interactions with proteins and microbial cells. Surface functional groups may be introduced that possess inherent antimicrobial activity and to which antimicrobial substances can be attached or coupled.

Jansen et al [45] described how the radiation modification of polyurethane by the hydrophilic substance, hydroxyethyl methacrylate, rendered the polyurethane surface hydrophilic and significantly reduced the adhesion of two coagulase-negative staphylococcal strains. Bridgett et al [46] also showed that coating a silicone cerebrospinal shunt catheter with Hydromer, a hydrophilic material which creates a hydrophilic layer on the shunt surface, significantly reduced the adherence of five strains of *S. epidermidis* and one strain of *S. aureus*. The authors suggested that the reduction in adherence may have been due to a weakening of hydrophobic interactions between the polymer surface and the bacterial cell surface.

Humphries et al [47] described how the modification of a series of surfaces using graft copolymers prevented bacterial adhesion. The copolymers had polyethylene glycol (PEG) side-chains and a backbone that was either uncharged, acidic, basic or amphoteric. The greatest reduction in adhesion was achieved with copolymers containing the most charged groups in the polymer backbone. The graft copolymers were considered to be adsorbed onto the surfaces via their backbone in such a way that the orientation of the PEG side-chains formed a steric barrier which inhibited bacterial adhesion. Similarly, Bridgett et al [48] showed that the adherence of three clinical isolates of *S. epidermidis* to polystyrene surfaces was significantly reduced when the surface was modified using a series of 16 Pluronic surfactants. They suggested that the adsorption of surfactants on the polystyrene surface created a sterically stabilized surface which conferred non-specific anti-adhesive properties. In related work, Desai and Hubbell [49] modified polyethylene tereph-

thalate films using poly(ethylene oxide). They found that adherence of *S. epidermidis*, *S. aureus* and *Ps. aeruginosa* was significantly less on the surface-modified films and they believed that this was due to a reduction in fibrinogen adsorption to these poly(ethylene oxide)-treated surfaces.

The cationic surfactant tridodecylmethylammonium chloride (TDMAC) has been used to bind antibiotics to catheter surfaces. For example, Trooskin et al [50] showed that the binding of penicillin to peritoneal catheters pre-treated with TDMAC significantly reduced adherence of *S. aureus*. Similarly, Kamal et al [51] described how the binding of cefazolin to TDMAC pre-treated intravascular catheters significantly reduced infection in situ.

Incorporation of antimicrobial agents

Possibly the most effective way of inhibiting bacterial growth is by incorporating antibacterial agents either directly into the polymer, or into a coating which can then be applied to the polymer. If such polymer/antimicrobial agent-systems are brought in contact with an aqueous medium, the drug should be released, providing a high local concentration surrounding the polymer. These devices should be able to prevent bacterial adhesion to the polymer surface and eradicate any adherent organisms present on it, thereby preventing further colonization and infection (Fig. 3).

To date, the incorporation of antimicrobial agents, in particular antibiotics, into hydrogel-coated medical devices has been conducted very much on an ad hoc basis by many clinicians. A variety of antibiotics and antibacterial agents have been used in this manner, with mixed results. The first attempt to incorporate an antibiotic into a catheter was described by Lazarus et al [52], wherein cephalothin was imbibed into Hydron, a hydrophilic polymer, which was then used to coat the surface of latex Foley catheters. A significant reduction in bacteriuria was observed with the cephalothin-releasing catheters, compared to control catheters, in a clinical trial involving 60 patients. Unfortunately, other clinical studies to determine the efficacy of the coated catheter concluded that it did not significantly reduce bacteriuria and was, therefore, of no potential benefit to the patient [53, 54]. More recently, Huaijin [55] described a silicone catheter with kanamycin incorporated into the tip. A significant reduction in the associated infection rate resulted with this tip and it was also reported that therapeutic levels of kanamycin were maintained in the urine for a period of 20–24 days post-insertion. Jansen et al [56] have shown that by coating polyurethane intravascular catheters with Hydrocath, a hydrophilic poly-*N*-vinylpyrrolidone coating, an antibiotic may also be incorporated. Teicoplanin loaded into the catheter was shown to be released in large amounts for up to 5 days. Teicoplanin-loaded catheters eliminated

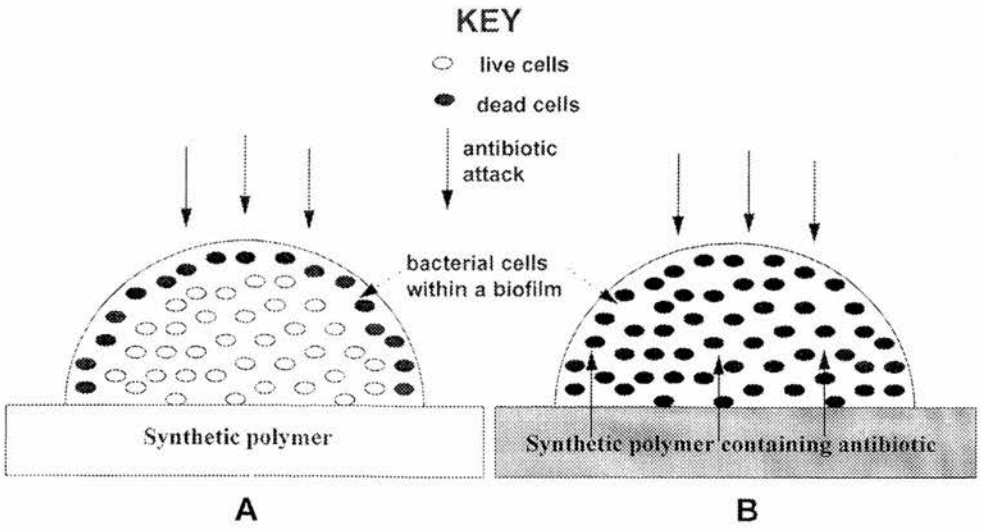

Fig. 3. Schematic diagram showing the effect of (A) conventional antibiotic therapy, and (B) an antibiotic-containing polymer.

adherent bacteria, thereby preventing catheter colonization for at least 48 hours.

The amount of antibiotic incorporated into the polymer and its release rate has recently been shown to be important. Schierholz et al [57], investigating rifampicin incorporation into cerebrospinal fluid shunts, concluded that foreign body infection could be prevented only if the drug were released in the environment of the implant over a long period of time and at a concentration exceeding the minimum bactericidal concentration of the infecting organism.

There are conflicting reports as to the effectiveness of ciprofloxacin-loaded catheters in preventing bacterial adherence and subsequent infection. Reid et al [58] showed in vitro that pre-incubation of silicone catheters with ciprofloxacin resulted in a 99% reduction of adherent *Ps. aeruginosa* cells in comparison with the reduction on control catheters. However, in another study Stickler et al [59] showed that if *Ps. aeruginosa* was suspended in urine, equal colonization of both ciprofloxacin-loaded and control catheters occurred.

Antibiotics have also been incorporated into polymethylmethacrylate bone cement to prevent infection developing after a primary joint replacement. Buchholz et al [60] reported that incorporation of antibiotics into the bone cement significantly reduced the risk of infection after a total hip replacement.

A variety of antimicrobial agents other than antibiotics have been examined in an attempt to prevent microbial adherence to urinary catheters. An in-vitro study has shown that silver-coated catheters are effective in preventing *Ps. aeruginosa* adherence [61]. The result of this work has been confirmed by the results of several clinical trials. Liedberg et al [62] showed that, in a trial involving 90 patients, bacteriuria after 5 days catheterization was significantly less in patients who had silver-coated catheters inserted. In another clinical trial, a silver oxide-coated catheter was shown significantly to lower the incidence of urinary tract infection in women not receiving antimicrobial therapy [62]. The antimicrobial activity of silver is thought to be due to the release of silver ions which have a broad spectrum of activity, which includes anaerobic organisms. It has been suggested that the silver ions act by binding to bacterial DNA or to the bacterial membrane [61]. Other agents which have been shown to be effective in preventing bacterial adherence to biomaterials include non-steroidal anti-inflammatory drugs, methyl and propyl parabens, iodine, tantalum and chlorhexidine in conjunction with silver sulphadiazine.

CONCLUSION

Bacteria are highly adaptive organisms, and implanted devices provide attractive surfaces for colonization with a subsequent increased risk of device-associated infection. Therefore, as Gristina concluded, 'Biomaterial surfaces must be modified to improve compatibility and tissue integration and to resist microbial colonization in the race for the surface' [1].

As described herein, biomaterial surface modification can effectively reduce bacterial adhesion; however, it remains unclear which strategy is most effective. Several studies have shown that physicochemical modification of a biomaterial surface can effectively reduce bacterial adhesion [44–46]. On the other hand, Reid et al [31] suggested that this approach was inappropriate as the modified biomaterial surface would be rapidly coated by a conditioning film on insertion in the body, thus altering the surface characteristics again. Harkes et al [63] also concluded that physicochemical modification of the surface would not prevent bacterial adhesion and suggested that incorporation of antimicrobial agents directly into the polymer would be more effective. Further current research involves the use of techniques such as ion implantation and chemical vapour deposition to create surfaces which promote macromolecular and tissue integration, rather than allowing microbial colonization. In addition, the coating of biomaterials with healthy tissue cells prior to implantation may promote tissue integration and prevent bacterial adhesion; however, the success or otherwise of such a strategy would be dependent upon the virulence determinants of the infecting bacteria.

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VARIATION OF *BACTEROIDES FRAGILIS* SURFACE STRUCTURES

S. Patrick

Department of Microbiology and Immunobiology, School of Clinical Medicine, Queen's University of Belfast, Royal Victoria Hospital Site, Grosvenor Road, Belfast BT12 6BN, UK. Tel: +44-1232-240503, FAX: +44-1232-439181, email: S.Patrick@QUB.ac.uk.

ABSTRACT

Bacteroides fragilis is a component of the normal commensal colonic flora of all adult humans and is the most common Gram negative obligately anaerobic bacterium isolated from clinical specimens. Within an individual strain three encapsulating surface structures of different morphologies can be observed by electron microscopy, namely a large capsule, small capsule and electron dense layer. Although morphologically distinct, the large capsule and electron dense layer have shared polysaccharide epitopes, which are absent in the small capsule. High molecular mass polysaccharide specific monoclonal antibody-labelling of populations enriched for either the large capsule or electron dense layer reveals antigenic variation within these populations. These high molecular mass polysaccharides are therefore not ideal targets for the immunodiagnosis of *B. fragilis* in clinical specimens. In contrast the common antigen may prove to be a suitable target.

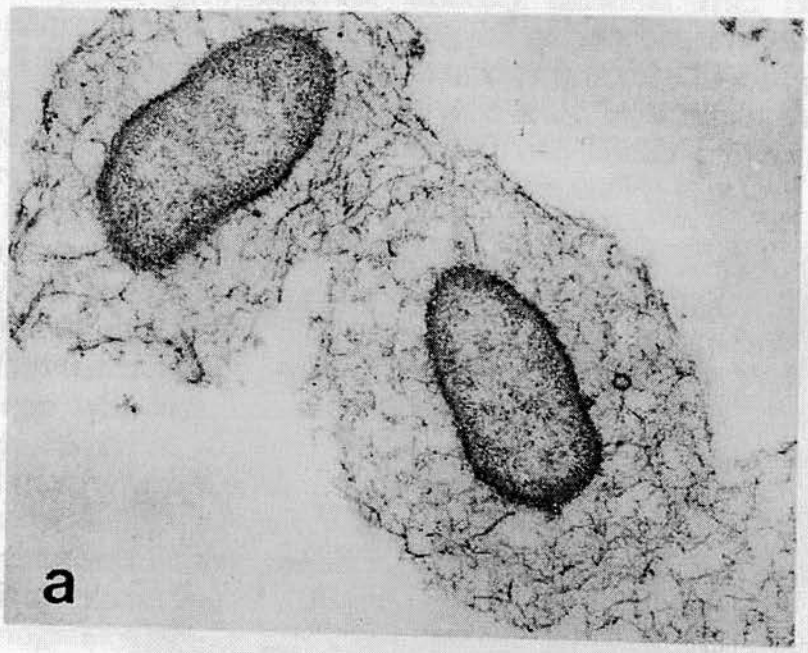
INTRODUCTION

Within-strain variation of bacterial surface structures, in terms of their presence or absence (*phase variation*), as well as *antigenic variation* of individual structures, are well documented phenomena in a number of pathogenic bacteria where it appears that this ability is directly linked with virulence [1,2]. Such variation enables the bacterium to cope successfully with the unique environment of the living host, which unlike other environments is not passive, but is actively trying to prevent colonisation with an array of defensive mechanisms. An understanding of the complexity of bacterial surface structure variation is important in relation to our understanding of the virulence of bacteria and therefore our long-term ability to treat disease. In the short-term, the identification of stable, as opposed to variable, antigens is necessary if the potential for the immunodiagnosis of bacteria using antibodies specific for surface structures is to be fully developed. Equally, where the generation of structural variation involves genetic rearrangements, the identification of stable DNA sequences is a necessity if genetic probes for virulence genes are to be used for bacterial identification.

Variation in the expression of capsules within individual strains of *Bacteroides fragilis* has been long recognised [3], although the implications of this with regard to the virulence of the bacterium and the potential for the immunodiagnosis of the bacterium have perhaps not been given due attention.

BACTEROIDES FRAGILIS PRODUCES THREE MORPHOLOGICALLY DIFFERENT CAPSULES

Bacteroides fragilis is capable of producing three morphologically different encapsulating surface structures: 1) a large capsule (LC), visible by both light and electron microscopy, 2) a small capsule (SC) visible by both light and electron microscopy and 3) an electron dense layer (EDL), only visible by electron microscopy (Figure 1). All of these structures may be produced by an individual *B. fragilis* isolate[4]. Populations can be enriched for these structures by Percoll step density gradient centrifugation on a gradient composed of 80%, 60%, 40% and 20% suspensions of Percoll[5]. Bacteria with large capsules remain on top of the gradient, bacteria with a small capsule are found at the 20-40% interface, and bacteria with an electron dense layer at the 60-80% interface. The bacteria at the 40-60% interface are a mixture of those with small capsules and those with an electron dense layer. The enriched populations retain their capsular types after sub-culture from the interface layers into broth culture overnight. Sub-cultures re-applied to the Percoll gradient band predominantly at the relevant interface and microscopy reveals the enriched populations.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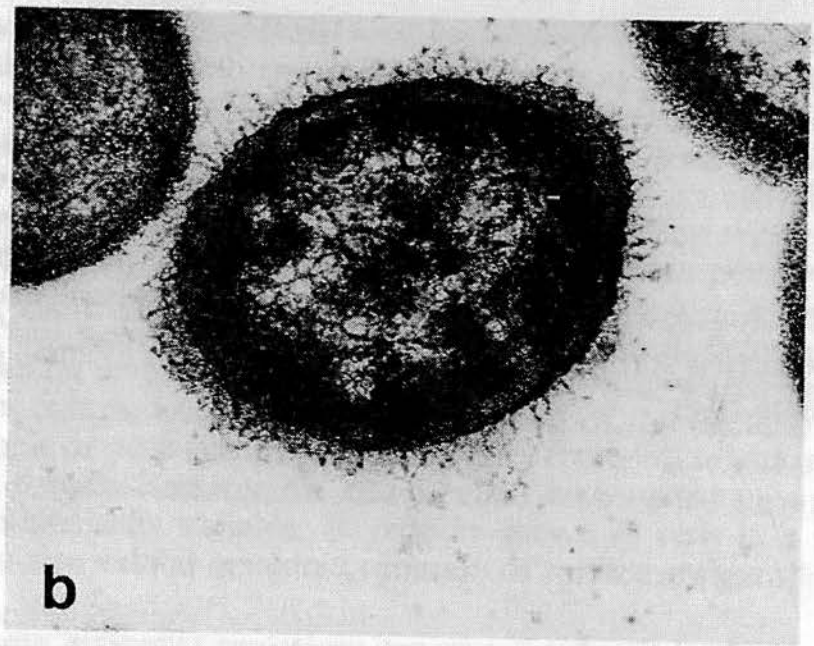
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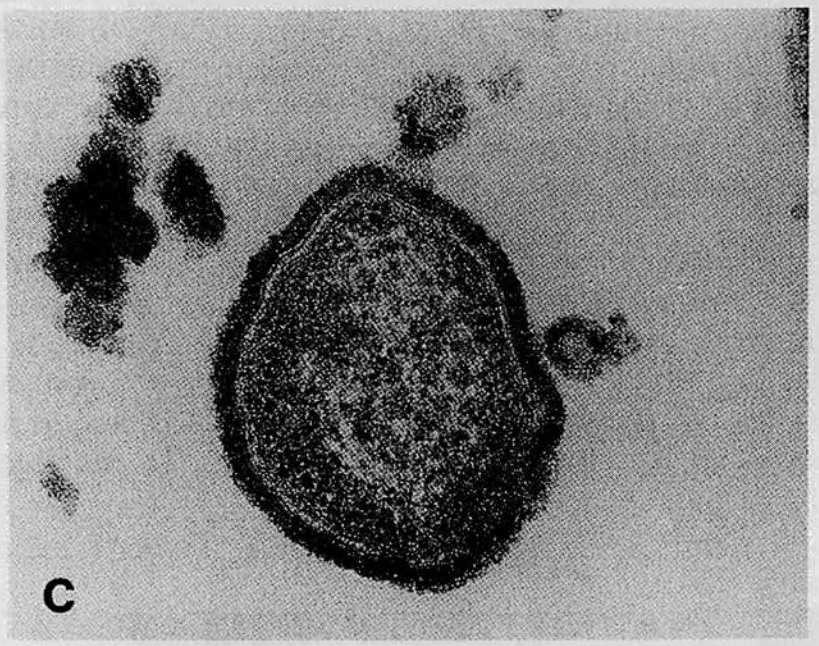

Fig. 1 Electron micrographs of ultrathin sections of *Bacteroides fragilis* illustrating encapsulating surface structures a) large capsule, b) small capsule and c) electron dense layer.

BACTEROIDES FRAGILIS CAPSULES ARE ANTIGENICALLY VARIABLE

Monoclonal antibodies (MAbs), specific for high molecular mass polysaccharides, give different labelling patterns after polyacrylamide gel electrophoresis and immunoblotting of polysaccharide extracts (Figure 2). MAb QUBf5 labels a ladder which is similar to the O-antigen of other Gram negative bacteria. Other MAbs give a wide high molecular mass band and some (e.g. QUBf7) have an associated fine ladder pattern, characteristic of polysaccharides with varying numbers of repeating units.

Techniques such as immunogold electron microscopy, of both ultrathin section and negatively stained whole cells, immunofluorescence microscopy and flow cytometric analyses have shown antigenic differences both between and within the morphologically different capsule types. The small capsule has an epitope which is absent from the LC and EDL[6]. The LC and EDL, although morphologically different, have shared epitopes. Labelling of populations homogeneous for the LC and EDL has also revealed antigenic variation within these populations [7]. The proportion of bacteria labelled within the population can be quantified either by microscopy or flow cytometric analyses. Different Mabs label different proportions of morphologically similar populations (Table).

To determine if antigenic variation is a widespread phenomenom in *B. fragilis*, twenty-five pus samples, from a range of anatomical sites, were examined for their reactivity with 7 MAbs[8]. The pus sample was labelled and examined by fluorescence microscopy, as was the *B. fragilis* isolate obtained from the specimen. Epitopes associated with the large capsule, small capsule and electron dense layer were each detected in the infected pus. Different pus samples were reactive with different groups of the 7 MAbs and there was no apparent relationship between labelling with a particular MAb and a particular anatomical site. If a pus sample was positive for a MAb, the corresponding isolate was also positive. However, in a few instances, the pus sample was negative and the pure culture was positive for a particular MAb. The labelling revealed antigenic heterogeneity both within the pus sample and the pure culture isolates; only a proportion of the bacteria in an individual pus sample or pure culture were labelled. Three blood cultures from patients with *B. fragilis* bacteraemia also labelled with varied groups of MAbs and were antigenically variable. *B. fragilis* grown *in vivo* in a mouse model of infection also exhibit antigenic variation of surface antigens[9].

How these antigenic variations are generated within the polysaccharides of *B. fragilis* remains to be determined. Interestingly, it is known that very small changes in the composition of a polysaccharide, for example the presence or absence of an acetyl group, is sufficient to change the antigenicity of a polysaccharide. Well-documented examples include K2a/K2ab, K18/K22 and

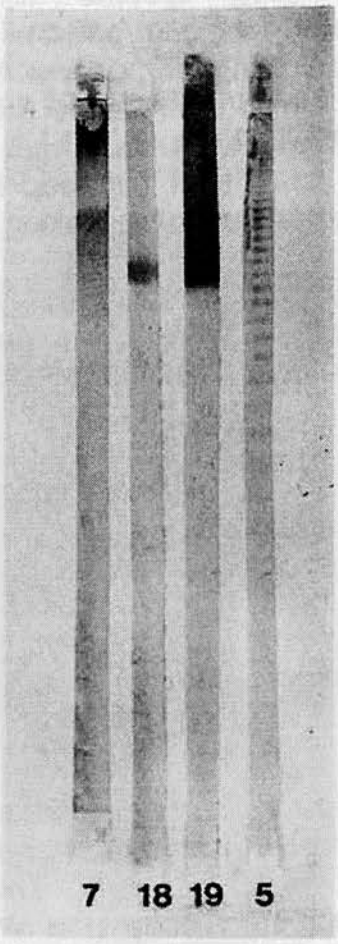

Fig. 2 Examples of murine monoclonal antibody labelling of immunoblots of *Bacteroides fragilis* polysaccharide extracts after PAGE. The blots were reacted with MAbs QUBF 7, 18, 19 and 5 followed by anti-mouse alkaline phosphatase conjugated antiserum. Note reactivity in the high molecular mass region and the ladder patterns associated with QUBF5 and 7.

Table 1. Proportion of *Bacteroides fragilis* NCTC9343 population labelled with murine monoclonal antibodies, as determined by flow cytometric analyses.

Population	Mean percentage of population labelled by monoclonal antibody:				
	QUBF4	QUBF5	QUBF6	QUBF7	QUBF8
Large capsule	9±5*	23±8	37±5	12±9	77±11
Small capsule	94±3	1±1	7±7	6±6	5±4
Electron dense layer	4±4	25±9	47±14	57±3	67±11

* standard error of mean

Table 2. Detection of *Bacteroides fragilis* and related species in pus samples

No. of samples	No. of culture positive pus samples	No. of common antigen positive pus samples**
147	*53(36%)	74(50%)

* 46 *B. fragilis*; 4 *P. melaninogenica*; 2 *Bacteroides sp.*; 1 *B. thetaiotaomicron*

** all of the culture positive samples were also positive for the common antigen, with the exception of one *B. fragilis* and one *Bacteroides sp.* culture positive sample

K1+/K1- capsular polysaccharides of *Escherichia coli* which only differ in the presence or absence of an O-acetyl group. These slight differences in the primary structure are sufficient to generate different epitopes[10].

The relationship between the variable high molecular mass polysaccharides and the polysaccharides A and B described by Pantosti and colleagues [11] remains to be determined. Inoculation of these polysaccharides into mice gives some protection against subsequent intraperitoneal challenge with *B. fragilis* (Tzianobos, this book). It would be interesting to investigate the bacterial populations in abscesses from the small number of mice, which although inoculated with the purified polysaccharides A and B, still develop abscesses.

Within and between strain antigenic variation of high molecular mass polysaccharides of *B. fragilis* is a phenomenon which seems to be widespread; it can be observed in culture collection strains and recent clinical isolates, during the course of natural infection and in a mouse model of infection. It therefore seem likely that antigenic variation is important in relation to the virulence of *B. fragilis*, particularly in relation to long-term infection.

IMMUNODETECTION IN PUS SAMPLES AND THE *BACTEROIDES FRAGILIS* COMMON ANTIGEN

Previous reports of commercially available kits for the immunodetection of *Bacteroides spp*, based on pooled polyclonal rabbit serum, indicated that they were not as successful as detection by culture[12-14]. The use of a MAb specific for the core region of the lipopolysaccharide was also investigated and again was less successful than identification by culture; this Mab only labelled *c.* 10% of the bacteria within a given strain[15].

As a result of the inherent variation of the high molecular mass polysaccharides and therefore the potential for low sensitivity of immunodiagnostic tests based on these antigens, the high molecular mass polysaccharides may not be ideal targets for the detection of *B. fragilis* in clinical samples with immunological probes. This could explain the lack of agreement between the detection of *B. fragilis* by culture and by immunodetection methods in the published literature. Therefore there is a need to identify a non-variable antigen common to *B. fragilis*. The common antigen of *B. fragilis*, first reported by Poxton and Brown in 1986 in the 7 strains they examined [16], may prove to be a suitable candidate and a better target for an immunodiagnostic test.

In relation to the high molecular mass variable antigens of *B. fragilis*, the common antigen is observed after polyacrylamide gel electrophoresis and

immunoblotting at a lower molecular mass position(Figure 3). By extraction of the common antigen and re-inoculation it is possible to obtain monospecific polyclonal antiserum specific for the common antigen. The potential use of the common antigen as a target for immunodetection was investigated using a monospecific polyclonal antiserum and immunofluorescence microsopy in two separate studies involving a total of 147 pus samples and 10 blood cultures[8].

Immunofluorescence labelling of 16 bacterial strains (including those enriched for the LC, SC and EDL, culture collection strains and recent clinical isolates obtained from Edinburgh, Amsterdam and Northern Ireland) with the anti-common antigen serum and Evan's Blue as a counter-stain, gave an average of 96% sensitivity with a minimum of 80%. *B. ovatus* and *B. thetaiotaomicron* labelled with a similar sensitivity, but lower intensity and *B. distasonis* was negative. The polyclonal antiserum also reacted with *Prevotella melaninogenica.*

Pus samples arriving in the laboratory were tested with the standard culture procedure which involved inoculation onto horse blood agar (ABA); anaerobic horse blood agar (BA); colistin-nalidixic acid agar (CNA); anaerobic blood agar plus 50μg/ml gentamicin (ABA+GM). Penicillin and gentamicin discs were applied to BA and CNA. Penicillin and metronidazole were applied to ABA+GM and gentamicin, penicillin and metronidazole to ABA. The anaerobes were identified with the API20A, ATB 32A and Mastring ID 8 systems. The pus samples were also processed for immunofluorescence microscopy using the polyclonal antiserum specific for the common antigen as a probe.

The results of comparing the standard clinical laboratory isolation procedures with immuno-labelling of the pus samples and immunofluorescence microscopy are summarised in Table 2. The specimens which were positive by culture for *B. fragilis*, *B. thetaiotaomicron*, *P. melaninogenica* or *Bacteroides sp*, with two exceptions, were also positive for the common antigen by immunofluorescence microscopy. A further 14% were positive for the common antigen and not by initial culture. Investigation of 12 of the specimens which were positive for the common antigen, but negative on initial routine testing in the laboratory, indicated that 6 of these labelled with some of the MAbs specific for the variable high molecular mass polysaccharides, thus confirming the presence of *B. fragilis* in the sample. A second attempt at culturing *B. fragilis* from these specimens after storage in the liquid nitrogen resulted in the isolation of *B. fragilis* from four samples. This study indicates that the use of the immunofluorescence technique alone would have underestimated the incidence of *B. fragilis* and related species by 1-2%, whereas with the current diagnostic laboratory culture methods it was underestimated by *c.* 14%.

Nine blood culture bottles, from which *B. fragilis* had been isolated, were also examined by immunofluorescence microscopy after labelling with the

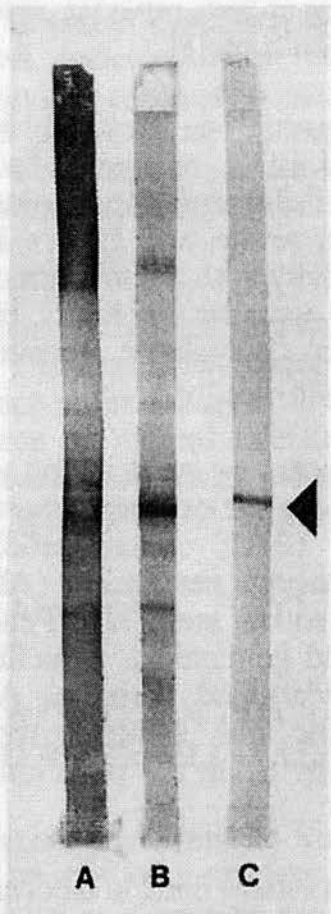

Fig. 3 Immunoblots of *Bacteroides fragilis* polysaccharide extracts after PAGE reacted with rabbit polyclonal antiserum followed by anti-rabbit alkaline phosphatase conjugated antiserum. a) antiserum prepared with whole cells of strain NCTC 9343, b) antiserum prepared with immunoblot purifed strain NCTC 9343 common antigen, c) antiserum prepared with immunoblot purified strain NCTC 9344 common antigen. Note lower molecular mass common antigen (arrow).

common antigen and these were all also positive for the common antigen. One blood culture which was positive for *B. distasonis* was negative for the common antigen.

CONCLUSION

It therefore appears that the common antigen, in contrast to the variable high molecular mass polysaccharides, has the potential to be a suitable target for the immunodetection of *B. fragilis* in clinical samples.

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Combined fluorescent in situ hybridisation and immunolabelling of *Bacteroides fragilis*

Gordon Ramage, Sheila Patrick *, Simon Houston

Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, RVH Site, Grosvenor Road, Belfast, BT12 6BN, Northern Ireland, UK

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Combined fluorescent in situ hybridisation and immunolabelling of *Bacteroides fragilis*

Gordon Ramage, Sheila Patrick *, Simon Houston

Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, RVH Site, Grosvenor Road, Belfast, BT12 6BN, Northern Ireland, UK

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Abstract

We have demonstrated that pure cultures of *Bacteroides fragilis* can be riboprobed with the oligoprobes BAC303 and EUB338, whilst simultaneously immunolabelled with either the mAb QUBF7, or polyclonal antiserum specific for a common antigen of *B. fragilis*. We were also able to distinguish between pure cultures of *B. fragilis* and *Escherichia coli*, by means of combined immunolabelling and riboprobing. The success of the combined technique is critically dependent on the size of the bacterial capsules, bacterial growth phase, antibody diluent and the length of the washing steps. The combined FISH and immunolabelling of bacteria has potential applications in studies of bacteria of medical and veterinary importance, as well as bacteria from other environments, as it yields information about both the identity and antigen expression of individual bacterial cells.

Keywords: rRNA; Oligonucleotide probe; Immunolabelling; Bacterial identification

1. Introduction

The potential for replacing or augmenting culture-dependent methods of bacterial identification by rapid immunological or molecular diagnostic methods has been the subject of many studies (e.g., Greisen et al., 1994; Patrick et al., 1995) as routine identification by culture is time consuming and costly. In particular the detection of obligately anaerobic bacteria in clinical samples by culture alone can considerably underestimate the true incidence of infection (Patrick et al., 1995). Such detection methods are also potentially useful in studies of the virulence of pathogenic bacteria.

With respect to molecular methods for bacterial identification, our understanding of bacterial taxonomy and ecology and our ability to identify bacteria has been considerably enhanced through the application of 16S and 23S rRNA oligonucleotide probes (Greisen et al., 1994; Roller et al., 1994; DeLong and Shah, 1990; Greisen et al., 1994; Poulsen et al., 1994; Amman et al., 1995). 16S and 23S rRNA sub-units fold in a precise manner to form ribosomes

Abbreviations: cAg, common antigen of *B. fragilis*; DAPI, 4′,6′-diamidino-2-phenylindole; EDL, electron dense layer; FISH, fluorescent in situ hybridisation; FITC, fluorescein isothiocyanate; HB, hybridisation buffer; LC, large capsule; mAb, monoclonal antibodies; PBS, phosphate buffered saline; PCR, polymerase chain reaction; rRNA, ribosomal RNA; SC, small capsule; TRITC, trimethylrhodamine isothiocyanate

* Corresponding author. Tel.: +44-012-322-405-03 ext. 2739; fax: +44-012-324-391-81; e-mail: s.patrick@qub.ac.uk.

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(Relman, 1993). The nucleotide sequences of the rRNA molecules contain conserved and variable regions, thus making both the rRNA and corresponding DNA gene sequences ideal targets for taxonomic studies (Woese, 1987). The 16S and 23S subunits contain sequences of ribosomal nucleic acid that are evolutionarily conserved between taxonomic groups, whilst concomitantly exhibiting sequence variations between different organisms. Therefore, when different bacteria are phylogenetically close the rRNA sequences are less varied (Relman, 1993). This sequence conservation and variation can be used as a criterion of bacterial taxonomic classification (Woese, 1987; Amman et al., 1995). The rRNA sequences are used as templates to design complementary oligonucleotide probes, according to the required specificity, in order to probe and identify bacteria directly without prior culture (Amman et al., 1992; Greisen et al., 1994).

The identification of medically important and unculturable bacteria using a number of nucleic acid detection techniques has been described (Greisen et al., 1994; Amman et al., 1995). A range of complementary universal, genus or species-specific oligonucleotide sequences, which act as either primers for amplification of the bacterial rRNA genes, or as direct complementary probes for the rRNA molecule, have been utilised for PCR, dot-blot hybridisation, and FISH, in the detection and identification of bacteria present in clinical samples (Relman, 1993; Greisen et al., 1994; Poulsen et al., 1994; Raskin et al., 1995; Licht et al., 1996; Wilson et al., 1997). FISH not only permits the detection and identification of bacteria, but also allows direct visualisation of the cellular morphology and provides an indication of the physiological state of the bacterium.

Immunological detection of bacteria through the application of indirect immunofluorescence is well documented (e.g., Desmonts et al., 1990; Patrick et al., 1995). Bacteria within a clinical sample, for example blood, pus, or cerebrospinal fluid, can be immunolabelled as a means of location and identification of infecting bacteria.

Individually, immunofluorescence microscopy and FISH, using rRNA specific oligonucleotide probes, allow the detection of culturable, and also viable but not culturable bacteria, whilst providing both a rapid and specific identification.

The aim of this study is to investigate the potential for combining FISH and immunofluorescence microscopy of bacteria. In combination, these techniques could allow not only the identification of specific bacteria without prior culture, but also provide details of antigen expression, morphology and the physiological state of bacteria. This could be particularly useful in studies of bacterial virulence determinant expression in situ, as the culture of bacteria in artificial media may radically alter the expression of virulence determinants (Smith, 1990). Here we demonstrate that both FISH and immunolabelling can be performed concomitantly on the same bacterial cell.

2. Materials and methods

2.1. Bacterial culture and treatment

B. fragilis NCTC9343, electron dense layer (EDL) population, small capsule (SC) population, and large capsule (LC) population were grown in defined minimal medium (van Tassell and Wilkins, 1978) as previously described (Patrick and Larkin, 1993). *E. coli* O128 was grown in nutrient broth. The organisms were grown under anaerobic (Don Whitley Mk. III anaerobic cabinet, 80% N_2, 10% CO_2 and 10% O_2) and aerobic conditions, respectively, at 37°C for 18 h. The cultures were re-inoculated into 10 ml of defined minimal medium and nutrient broth, respectively, for 5 h until the bacterial populations had reached exponential phase growth to yield a bacterial density of 1×10^8 cfu per ml.

2.2. Removal of the large capsule

Sodium periodate (0.02 M) was prepared in sterile dH_2O. The LC population was centrifuged for 2 min at 14 000 g. The bacteria were then washed twice in $1 \times$ PBS (130 mM NaCl, 10 mM Na_2HPO_4, 10 mM $NaH_2PO_4.2H_2O$ [pH 7.2]) and the pellets retained. These were treated with sodium periodate and incubated for 1 h at 37°C in the dark. The bacteria were centrifuged at 14 000 g and subsequently washed twice in PBS. The cells were resuspended in PBS. The suspension was then adjusted to the same bacterial density as above. Bacteria, suspended in PBS as

above, were sonicated in an MSE 150 soniprep for 50 s at an amplitude of 5 microns and washed as above. Removal of the capsule was monitored by light microscopy of capsule smears (Patrick et al., 1996).

2.3. Bacteroides population separation

B. fragilis NCTC9343 was grown to late exponential phase and the three capsular populations separated by density gradient centrifugation using a Percoll gradient, as described by Patrick and Reid (1983).

2.4. Fluorescent in situ hybridisation (FISH)

FISH, in relation to time, temperature and buffer, was empirically optimised to maximise signal intensity (Stahl and Amman, 1991).

2.4.1. Slide preparation

12-well Teflon coated slides (ICN Biomedicals, Aurora, OH, USA) were soaked in Decon solution (1% v/v in dH_2O) for 1 h, washed in dH_2O and air dried. The slides were immersed in gelatin (0.1% w/v in dH_2O) and chromium potassium sulphate (0.01% w/v in dH_2O [Sigma, St Louis, USA]), removed and air dried.

2.4.2. Cell fixation and storage

B. fragilis and *E. coli* cells, at the appropriate density, were spotted onto each well of a slide in 10 μl volumes, under anaerobic and aerobic conditions, respectively, and air dried. The slides were then immersed in paraformaldehyde (4% w/v in 3 × PBS: 390 mM NaCl, 30 mM Na_2HPO_4, 30 mM $NaH_2PO_4.2H_2O$ [pH 7.2]), for 30 min at 4°C. The slides were removed, washed briefly with 1 × PBS and immersed in ice cold absolute ethanol for 10 min at −20°C. The slides were then removed, air dried and stored at −20°C for up to several months without any apparent deterioration of hybridisation signal.

2.4.3. Oligonucleotide probes

Three oligonucleotide probes were selected based on published findings, all numberings list the corresponding positions in the *E. coli* 16S rRNA. The following oligonucleotide probes were selected: BAC303 (5′-CCAATGGGGGACCTT-3′), a probe showing 100% similarity to 16S rRNA sequences of the genera *Bacteroides* and *Prevotella* at the positions 303–319 of the 16S rRNA (Manz et al., 1996); EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′), a eubacterial probe specific for all eubacteria and ranging from positions 338–355 on the 16S rRNA (Amman et al., 1995); and a eukaryotic oligonucleotide probe (5′-ACCAGACTTGCCCTCC-3′) ranging from positions 502–516 of 16S rRNA (Amman et al., 1995). The oligonucleotide probes were synthesised by Eurogentec (Abingdon, UK) and covalently linked at the 5′ end with a fluorescein marker, purified by HPLC and stored at −20°C in ddH_2O at a concentration of 50 $\eta g/\mu l$.

2.4.4. Pre-hybridisation

The slides were removed from storage and dried in a 37°C incubator. They were then washed in a series of ethanol washes (50%, 80% and 98% v/v in dH_2O) for 3 min each and then allowed to air dry.

2.4.5. Hybridisation

Hybridisation buffer (HB: 0.9 M NaCl, 20 mM Tris/HCl and 0.01% sodium dodecyl sulphate [pH 7.2]) at volumes of 8 μl was spotted onto each slide well, then 1 μl aliquots of each of the EUB338 and BAC303 oligonucleotide probes were applied to the buffer. The eukaryotic probe was used as a control to monitor non-specific oligoprobe binding. Filter paper (3M Whatman, Maidstone, Kent, UK) was placed inside a polypropylene screw cap tube (Corning, NY, USA), which acted as the hybridisation chamber, the paper was soaked with HB. The slide was placed in the tube and the cells hybridised in an incubator at 44°C for 3 h.

2.4.6. Post-hybridisation (FISH only)

The slides were rinsed with HB and then incubated for 1 h at 54°C in HB within a water bath. The slide was then rinsed in ddH_2O to remove salt precipitant, and dried at 37°C. The cells were counter-stained with 10 μl (10 $\mu g/ml$) of 4′,6′-diamidino-2-phenylindole (DAPI) on each well and incubated at 4°C for 30 min. The DAPI stain was washed off the slide with 1 × PBS and allowed to air dry. The slides were then mounted in Citifluor (Agar

Scientific, Stanstead, Essex, UK), and the coverslip sealed with nail varnish.

2.4.7. Post-hybridisation (Combined FISH and immunolabelling)

The slides were briefly washed with a small volume of freshly prepared HB at 54°C, rinsed with ddH_2O and then dried at 37°C. Primary monoclonal antibody (mouse anti-*B. fragilis*, NCTC9343 EDL population, IgG2) QUBF7, produced from BALB/c ascites (Lutton et al., 1991), or polyclonal antisera (rabbit anti-*B. fragilis* common antigen (cAg) (Patrick et al., 1995)) was appropriately diluted in $1 \times$ PBS. The antibody was applied in 10 μl volumes to each well and incubated at 37°C for 20 min. The antibody was rinsed off quickly with HB and washed for 10 min in HB at 37°C. The slides were then removed from washing and the secondary antibody applied. The secondary antibodies (goat anti-mouse or goat anti-rabbit IgG trimethylrhodamine isothiocyanate (TRITC) conjugated [Sigma]) and DAPI were appropriately diluted in $1 \times$ PBS. The antibody/DAPI mixture, after application, was incubated and washed as above. The slides were then mounted and viewed as before. To control for non-specific antibody binding, wells without previous primary antibody incubation were incubated with secondary antibody.

2.4.8. Microscopic and photographic details

Slides were viewed using a Leitz Dialux 20 fluorescence microscope fitted with a WILD HPS 50 photoautomatic system. The microscope was fitted with filters for rhodamine or fluorescein and a triple blue/green/red filter. Photographs were taken on Fuji colour 400 ASA film.

3. Results

3.1. The effects of growth phase on the riboprobe signal intensity

Broth grown *B. fragilis* NCTC9343, EDL population, were sampled at one hourly time intervals post-inoculation (1–10 hours) and fixed onto the slides as described above. The bacteria were hybridised with the probes EUB338 and BAC303. It was demonstrated that the fluorescent signal intensity varied when the cells were fixed at different stages of growth. Bacteria fixed at early exponential phase (1–2 hours) showed very low levels of fluorescence intensity, as did bacteria in death phase (8–9 hours). Those bacteria fixed at late exponential phase (5 h) showed optimal fluorescence intensity (results not illustrated).

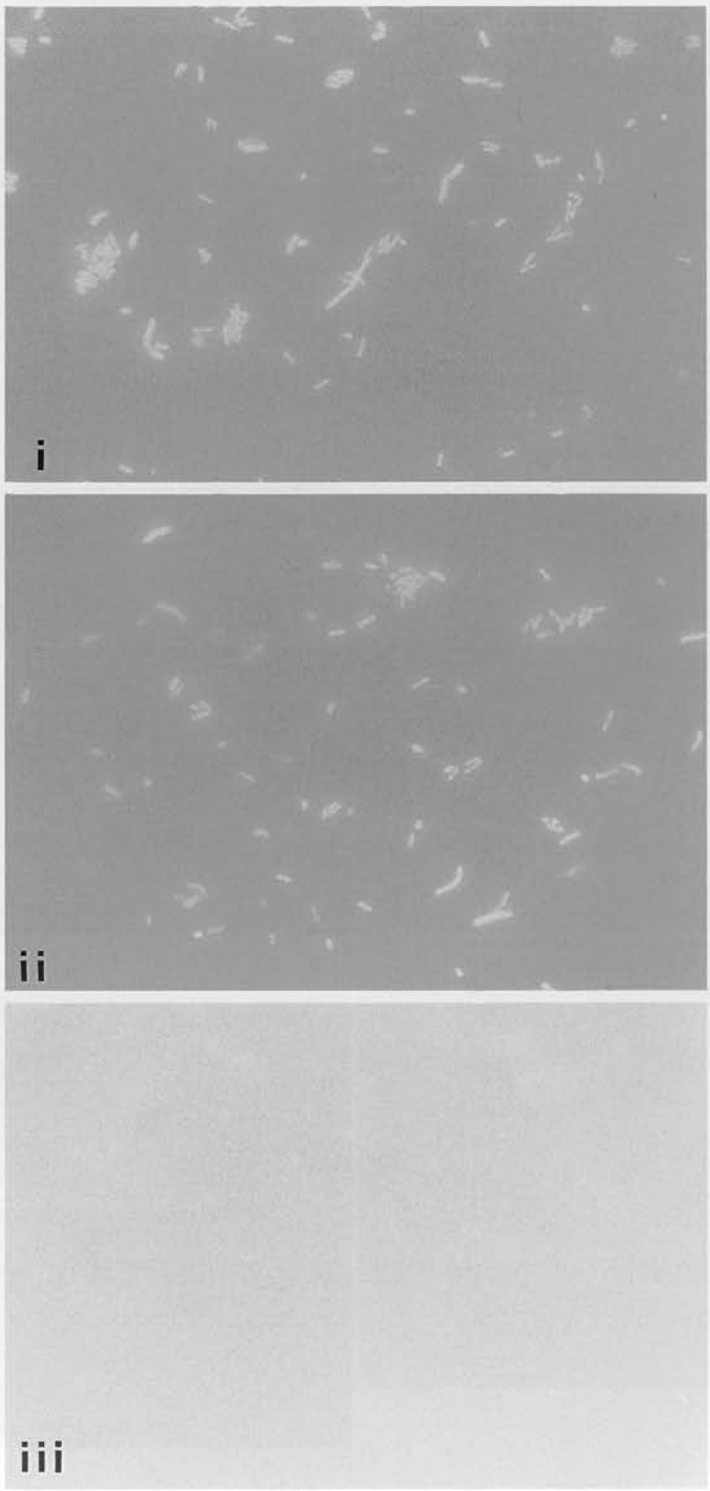
3.2. The effects of in situ hybridisation on B. fragilis capsular populations

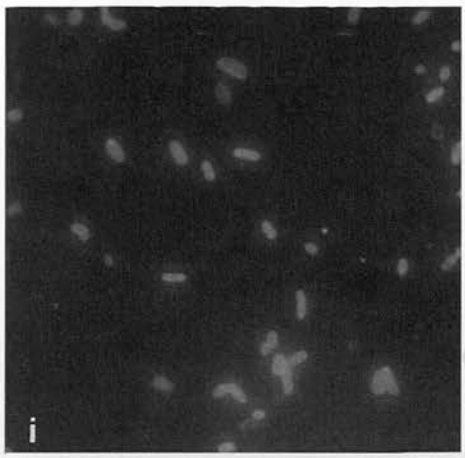
The three capsular populations were fixed as described above. Late exponential phase *B. fragilis* NCTC9343 population expressing a large capsule (LC), small capsule (SC) or narrow electron dense surface layer, only visible by electron microscopy (EDL), were hybridised with the EUB338 and BAC303 probes. Fig. 1 shows a weaker signal intensity in the LC (Fig. 1, iii) when compared with both the SC and the EDL. To investigate whether the capsular material was responsible for the poor signal observed, the capsule was removed by either sodium periodate treatment or mild ultrasonication. Capsule loss was monitored by negative-staining and microscopy. It was shown that signal intensity was improved after the LC was removed by either method. This suggests that the capsule may be responsible for the poor hybridisation signal obtained.

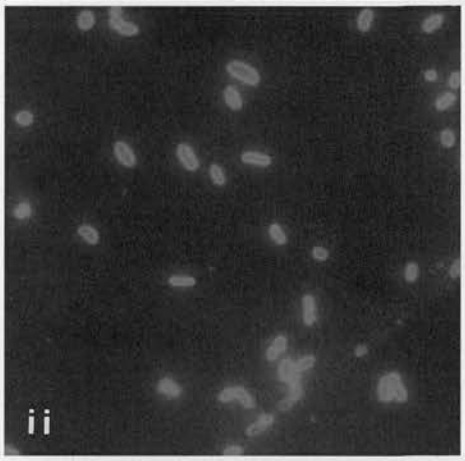
3.3. Combined FISH and immunolabelling on B. fragilis

When the optimised FISH was combined with the immunolabelling method described by Patrick and Larkin (1993) a low riboprobe fluorescent signal intensity was observed. This suggests that the probe

Fig. 1. Micrographs of *B. fragilis* NCTC9343, EDL population, SC population and LC population, hybridised with probes EUB338 and BAC303 and viewed with a fluorescein filter: (i) EDL population; (ii) SC population; and (iii) LC population ($\times$100). Note that the fluorescent signals of the EDL and SC populations are markedly more intense than the fluorescent signal of the LC population.


i
ii
iii





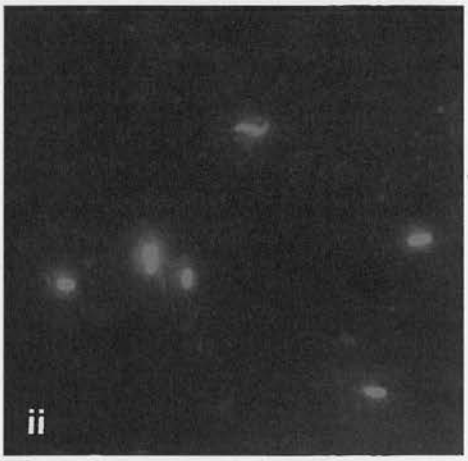


Fig. 3

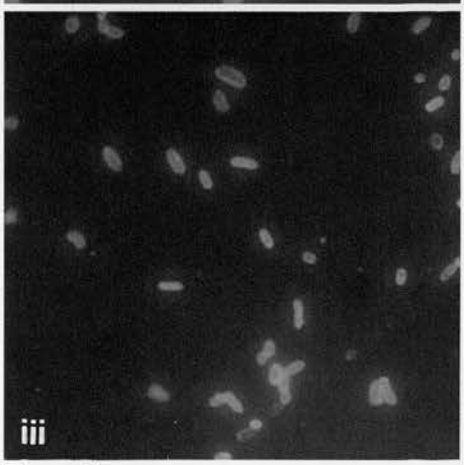


Fig. 2

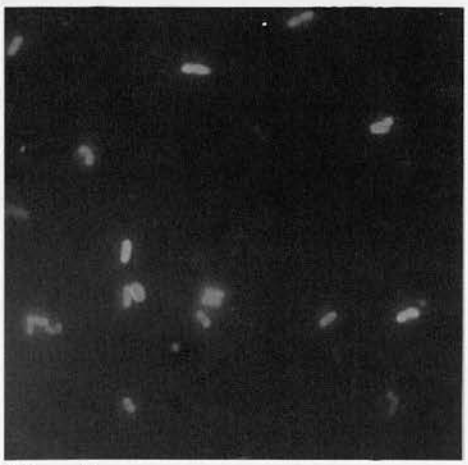


Fig. 4

had become dissociated from the rRNA and leached from the cell during immunolabelling, giving a poor signal. It was identified that the signal was being lost during the 30 min washing steps and also when the primary mAb was undiluted hybridoma culture supernatant. Therefore, by reducing the washing times to 10 min and using either mAb produced in ascites fluid or polyclonal antisera, diluted in PBS the loss of the riboprobe fluorescent signal was minimised.

B. fragilis NCTC9343, EDL population, first hybridised with the BAC303 and EUB338 probes, followed by immunolabelling with either mAb QUBF7 or polyclonal antiserum specific for the *B. fragilis* cAg is illustrated in Figs. 2 and 3. The combined labelling permitted simultaneous specific identification of the *B. fragilis*. In the case of the anti-cAg serum, all the bacteria within the population were labelled with both the antiserum and the oligoprobe (Fig. 2). In contrast, with mAb QUBF7 only a proportion of the population carried this epitope and were labelled Fig. 3.

3.4. Identification of B. fragilis in a mixed population of E. coli / B. fragilis using combined FISH and immunolabelling

An EDL population of *B. fragilis* NCTC9343 and a population of *E. coli* O128 were prepared as above, then mixed together at the appropriate cellular density, spotted onto slides and fixed as described above. Fig. 4 illustrates the combined labelling of the mixed bacterial population. The *B. fragilis* cells appeared yellow/orange in colour as a result of labelling with both the oligoprobe and the anti-cAg serum.

4. Discussion

The combined technique of FISH and immunofluorescence labelling can potentially improve our ability to detect and identify bacteria. We have demonstrated that labelling of a pure culture of *B. fragilis* could be performed with the 16S rRNA probes, EUB338 and BAC303, followed by labelling with either monoclonal antibodies or polyclonal antiserum. The technique was also applied to a mixed culture of *B. fragilis* and *E. coli*, and we were able to distinguish the two bacteria from each other by means of rabbit anti-*B. fragilis* common antigen specific polyclonal antiserum.

The success of the combined labelling technique is dependent on a number of critical factors. Firstly, as reported by others (e.g., Poulsen et al., 1993), the intensity of the fluorescent signal from the 16S rRNA probe was dependent on growth phase, with optimal fluorescent signal intensity observed in the exponential phase. This is thought to relate to the ribosomal content of the bacterial cell. Secondly, populations of bacteria with LC exhibited a reduced fluorescent signal with 16S rRNA probe labelling when compared to a population of the same bacterial strain which did not express the large capsule (EDL population). Removal of the LC with either sodium periodate treatment or mild ultrasonication prior to FISH resulted in fluorescent intensities similar to those of the EDL population. This suggests that the LC provides a barrier to penetration of the oligonucleotide probe, and that the differences are not due to different physiological states of the LC and EDL populations. This is in contrast to the results of Poulsen et al. (1993) who reported that, with *E. coli*,

Fig. 2. Micrograph of the same field of *B. fragilis* NCTC9343, EDL population, hybridised with probes EUB338 and BAC303 and immunolabelled with polyclonal antiserum specific for the *B. fragilis* common antigen: (i) viewed with fluorescent filter to show oligoprobe labelling; (ii) viewed with the rhodamine filter to show antibody labelling; and (iii) viewed with the red/blue/green filter to show combined labelling (×100). Note that the combined label permits the visualisation of a pure culture of *B. fragilis* with the oligoprobe, whilst simultaneously visualising expression of the *B. fragilis* common antigen.

Fig. 3. Micrographs of *B. fragilis* NCTC9343, EDL population, hybridised with probes EUB338 and BAC303 and immunolabelled with mAb QUBF7, (i) viewed with fluorescent filter to show oligoprobe labelling; (ii) viewed with the rhodamine filter to show antibody labelling (×100). Note that the epitope recognised by mAb QUBF7 is not expressed by all of the bacteria in the population.

Fig. 4. Micrographs of a mixture of *B. fragilis* NCTC9343 EDL population and *E. coli* O128 hybridised with probes EUB338 and BAC303, immunolabelled with anti-*B. fragilis* common antigen polyclonal antiserum and viewed with a red/blue/green filter (×100). Note the yellow/orange colour of the *B. fragilis* which are labelled by both the oligoprobe and the antiserum (×100).

the fluorescent signal intensity was altered only by the ribosomal content of the cell and not the nature of the outer layers of the bacterium. Finally, in relation to fluorescent immunolabelling, post-oligoprobing, the prolonged washing procedure in PBS normally adopted for immunolabelling (Patrick and Larkin, 1993) reduced the fluorescent labelling of the oligoprobes, as did the use of undiluted hybridoma cell culture supernatant as the primary antibody. Therefore, the length of the washing steps was reduced, such that they were sufficient to prevent non-specific immunolabelling while retaining oligoprobe signal intensity. The washing steps were carried out in hybridisation buffer rather than PBS. The reduction in oligoprobe signal resulting from the use of undiluted hybridoma cell culture supernatant for the primary antibody may have occurred because the high pH or the composition of the hybridoma growth medium promoted dissociation of the probe. Filter concentrated mAb, ascites fluid or polyclonal antisera diluted in PBS appeared to have no detrimental effect on the oligoprobe labelling.

As a research tool, combined FISH and immunolabelling provides the opportunity to concomitantly investigate a variety of parameters. For example, the complexities of bacterial populations in biofilms may be investigated with respect to the interactions of bacteria in situ, whilst also exploring whether the physiological state and morphology vary. Furthermore, the bacterial population may be monitored for antigenic variation. The technique may potentially be applied to studies of clinical material, models of infection and other environmental samples. In particular, the ability to not only specifically identify bacteria in situ, but monitor virulence determinant expression without culture is particularly useful in studies of bacterial pathogenesis as the characteristics of bacteria growing in vivo may differ considerably from those growing in vitro (Smith, 1990).

The application of the combined FISH and immunolabelling technique directly to clinical detection and diagnosis of bacteria is more problematic, given the dependence of fluorescent signal on the physiological state of the bacterium and the potential for surface structures such as capsules to restrict oligoprobe entry. These factors do not present a difficulty when detecting bacteria by immunolabelling, provided the target antigen is known to be stably expressed, is common to all strains, and is present on the bacterial surface (Patrick, 1997).

As flow cytometry has been used to detect fluorescently labelled bacteria after either immunolabelling (Lutton et al., 1991) or FISH (Bauman and Bentvelzen, 1988), there is also the potential for flow cytometric analysis of bacteria labelled by a combination of the techniques.

The amplification of rRNA gene sequences by PCR as a means of detecting bacteria in clinical samples has been described (Greisen et al., 1994). Although PCR amplification of rRNA gene sequences can provide a rapid positive identification of bacteria, this technique does not, however, provide information pertaining to the morphology, physiological activity and antigenic character of the bacteria. We are currently in the process of assessing and comparing PCR of the rRNA gene and combined FISH and immunolabelling for bacterial detection in clinical samples.

While the ability to rapidly identify bacteria within clinical samples without prior culture is attractive, both in terms of the potential speed with which positive identification can be made and the possibility of detecting bacteria which may be non-culturable, it is likely that in the short term such techniques will remain as a supplement to culture methods. This is particularly the case where the antibiotic sensitivity profiles of the bacteria are required. In the longer term, if problems relating to oligoprobe signal intensity are overcome, then one of the major advantages of labelling the whole bacterial cell rather than extracted DNA is the ability to correlate the results with bacterial cell morphology and thus reduce the chance of false-positive results, an attribute not demonstrated by PCR.

5. Conclusion

The combination of FISH and immunolabelling of bacteria provides a potentially useful research tool in the context of both the studies of bacterial pathogenesis and general studies of environmental bacteria, as it permits concomitant in situ identification of bacteria and information pertaining to surface antigen expression.

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Improved detection of infection in hip replacements

A CURRENTLY UNDERESTIMATED PROBLEM

Michael M. Tunney, Sheila Patrick, Sean P. Gorman, James R. Nixon, Neil Anderson, Richard I. Davis, Donna Hanna, Gordon Ramage

From the Queen's University, Belfast, Northern Ireland

Our aim was to determine if the detection rate of infection of total hip replacements could be improved by examining the removed prostheses. Immediate transfer of prostheses to an anaerobic atmosphere, followed by mild ultrasonication to dislodge adherent bacteria, resulted in the culture of quantifiable numbers of bacteria, from 26 of the 120 implants examined. The same bacterial species were cultured by routine microbiological techniques from only five corresponding tissue samples. Tissue removed from 18 of the culture-positive implants was suitable for quantitative tissue pathology and inflammatory cells were present in all samples. Furthermore, inflammatory cells were present in 87% of tissue samples taken from patients whose implants were culture-negative. This suggests that these implants may have been infected by bacteria which were not isolated by the techniques of culture used.

The increased detection of bacteria from prostheses by culture has improved postoperative antibiotic therapy and should reduce the need for further revision.

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M. M. Tunney, PhD, Research Fellow
S. Patrick, PhD, Senior Research Officer
D. Hanna, BSc, Research Student
G. Ramage, BSc, Research Student
Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, Royal Victoria Hospital Site, Grosvenor Road, Belfast BT12 6BN, UK.

M. M. Tunney, PhD, Research Fellow
S. P. Gorman, PhD, Professor of Pharmaceutical Microbiology
School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK.

J. R. Nixon, FRCS, Consultant Orthopaedic Surgeon
Withers Orthopaedic Centre, Musgrave Park Hospital, Stockmans Lane, Belfast BT9 7JB, UK.

N. Anderson, MRCPath, Consultant Pathologist
The Royal Group of Hospitals and Dental Hospital Health and Social Services Trust, Grosvenor Road, Belfast BT12 6BA, UK.

R. I. Davis, MRCPath, Senior Registrar in Pathology
Belfast City Hospital, Lisburn Road, Belfast BT9 7BL, UK.

Correspondence should be sent to Dr S. Patrick.

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Despite the use of ultra-clean-air operating theatres and antibiotic-impregnated bone cement,[1] between 2%[2] and 15%[3] of all revision hip operations are because of infection of the implant.[4] Unfortunately, after revision the rate of infection is higher than after primary procedures, with as many as 40% of revised hips becoming reinfected.[5] This has been attributed to the presence of unrecognised infection at the time of the first revision,[5] possibly because bacteria colonising the surface of implants grow predominantly in adherent biofilms[6] and may not be detected by aspiration or by standard culture techniques.[7] In addition, the well-proven incidence of anaerobic bacteria in joint infection[8] emphasises the need for special methods of culture.

We aimed to improve the isolation of bacteria from hip prostheses removed at revision operations by using mild ultrasonication to dislodge bacteria adhering to the surface of the implant, and strict anaerobic techniques.

Patients and Methods

From March 1996 to May 1997 we retrieved 120 hip prostheses from patients at revision operations. There were 70 women and 50 men with a mean age of 69 years (27 to 92). The mean interval from the previous joint replacement to the revision operation was 8.8 years (4 months to 24 years). All had standard preoperative care.

The skin was painted with Betadine (Seton Healthcare, Oldham, UK) and the incision area covered with an adhesive plastic drape. All operations were carried out with vertical laminar air flow and the operating team wore disposable impervious drapes. Routine antibiotic prophylaxis consisted of 2 g of cefamandole (Kefadol; Dista Products Ltd, Basingstoke, UK) given intravenously at the time of the induction of anaesthesia. Further doses of 1 g of cefamandole were given at eight and 16 hours after surgery. Cefamandole is recommended by the British National Formulary for surgical prophylaxis since it is a broad-spectrum antibiotic for both Gram-positive and Gram-negative bacteria. The removed femoral and acetabular components were placed aseptically in separate sterile bags. Tissue in contact with the implants was also removed and placed in sterile bottles. All specimens were immediately placed in an anaerobic jar for transport to an anaerobic cabinet.

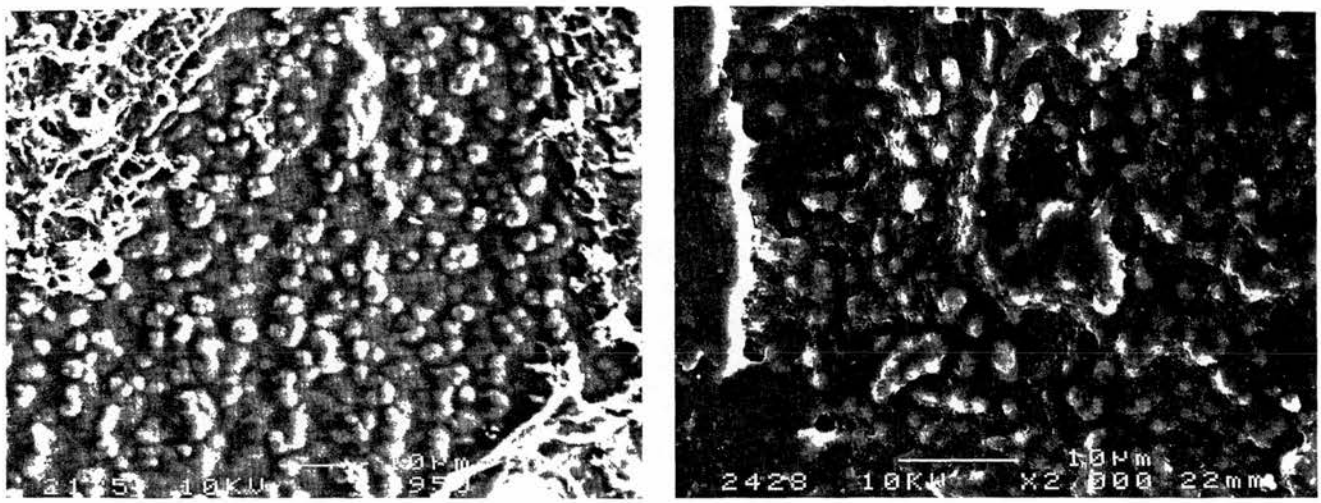

Fig. 1

SEM showing the bacterial biofilm on a retrieved orthopaedic implant viewed at a magnification of (a) ×700 and (b) ×1500.

high-power field: 0, absent; 1, 1 to 10 cells; 2, 10 to 20 cells; or 3, 20 or more cells.

Results

Quantifiable numbers, from 2×10^6 to $>10^6$, of bacteria were cultured from 26 of the 120 samples examined (22%). The infection in seven of the samples was caused by a single *Staphylococcus* species. Three other samples were infected by a combination of two *Staphylococcus* species. The anaerobic bacterium, *Propionibacterium acnes,* was isolated as the single infecting organism in 12 samples and a further four samples were infected by a combination of *P. acnes* and an aerobic Gram-positive coccus (Table I); 16 of the 26 samples (62%) therefore involved *P. acnes*. SEM confirmed that bacteria were able to grow within a confluent biofilm on the surface of the prosthesis (Fig. 1). Organisms similar to those isolated from the implants were cultured from only five of the 26 corresponding tissue samples. All the other tissue samples were culture-negative. No bacteria were isolated from the 20 control prostheses.

Tissue samples from 18 of the 26 culture-positive implants were suitable for histological examination and inflammatory cells were present in all 18 samples (Table I). Lymphocyte and macrophage infiltration was more extensive than polymorphonuclear leucocyte infiltration with 17 samples having lymphocyte and macrophage scores of 1 to 3 compared with only ten with polymorphonuclear leucocyte scores of 1 to 3 (Table II).

Tissue samples from 63 of the 94 culture-negative implants were also suitable for histological examination; inflammatory cells were present in 55 (87%). Lymphocyte and macrophage infiltration was again more extensive than polymorphonuclear leucocyte infiltration. Of the 55 tissue samples, 52 and 54 had lymphocyte and macrophage scores of between 1 and 3, respectively, whereas only 31 samples had a similar level of polymorphonuclear leucocyte infiltration (Table II).

We examined the hospital records of 18 of the 26 patients with culture-positive implants and of 52 of the 94 patients with culture-negative implants. Of the 18 culture-positive patients, 12 had a preoperative diagnosis of either aseptic loosening or dislocation, and the other six patients had been suspected of having infection. In four of these six patients, however, no bacteria had been cultured from either joint fluid aspirated preoperatively or from tissue samples removed at the time of surgery. Preoperative aspiration of joint fluid in the other two patients resulted in the isolation of *Staphylococcus epidermidis* (case 4) and *S. aureus* (case 7). Similar organisms to those isolated preoperatively were cultured from the implants after sonication and from the surrounding tissue after homogenisation. Of the 52 patients with culture-negative implants, 50 had a preoperative diagnosis of either aseptic loosening or dis-

Table II. The relationship between inflammatory cell presence and bacterial isolation by culture

	Inflammatory cell score											
	PMN*				LYM†				MAC‡			
	0	1	2	3	0	1	2	3	0	1	2	3
Number of culture-positive implants	8	6	4	0	1	10	7	0	4	4	6	7
Number of culture-negative implants	32	24	5	2	11	17	30	5	9	8	15	32

* polymorphonuclear leucocyte
† lymphocyte
‡ tissue macrophage

could therefore have resulted from intraoperative seeding or haematogenous spread.

We found high inflammatory scores in 87% of the tissue samples from patients whose implants were culture-negative. Infiltration with lymphocytes and macrophages was predominant, which suggests that the implants removed from these patients may have also been infected by low-virulence organisms which were not isolated by the techniques used. Similar results were obtained by Fehring and McAlister[14] in a smaller study of nine patients in whom the histological evaluation was positive and the cultures negative; in six there was a high clinical suspicion of infection.

We believe that infection of retrieved hip prostheses is currently underestimated. We recommend that the removed implants are sonicated to dislodge adherent bacteria and then sampled directly to improve bacterial isolation under anaerobic conditions. The examination of tissue alone does not always result in the isolation of the causative organism. The improved detection and isolation allow the use of suitable antibiotics for a longer postoperative period, which should improve the clinical outcome. We are now using polyclonal antisera and monoclonal antibodies specific for the infecting bacteria and 16S rRNA oligonucleotide probes to increase the detection of non-culturable bacteria.

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Antimicrobial Susceptibility of Bacteria Isolated from Orthopedic Implants following Revision Hip Surgery

MICHAEL M. TUNNEY,[1,2] GORDON RAMAGE,[1] SHEILA PATRICK,[1] JAMES R. NIXON,[3] PHILIP G. MURPHY,[4] AND SEAN P. GORMAN[2]*

Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, Belfast BT12 6BN,[1] School of Pharmacy, The Queen's University of Belfast,[2] and Department of Bacteriology, Belfast City Hospital,[4] Belfast BT9 7BL, and Withers Orthopaedic Centre, Musgrave Park Hospital, Belfast BT9 7JB,[3] United Kingdom

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The susceptibilities of 49 isolates recovered from orthopedic implants to seven antimicrobial agents were evaluated by the broth microdilution method. Ciprofloxacin and vancomycin were more active than gentamicin, representing aminoglycosides which are routinely incorporated into bone cement, and also more active than the peroperative antimicrobial agents cefamandole and erythromycin. The use of ciprofloxacin and vancomycin in vivo, therefore, warrants further evaluation.

Total hip replacement has become commonplace in recent years because of the success of this procedure in restoring function to the affected joint (6). Unfortunately, bacterial infection has been a significant complication following this procedure, with implant infection implicated in 22% of revision operations in a recent study (12). Removal and replacement of the prosthesis are usually required to eradicate the infection, with attendant patient trauma and increased cost (1, 8). Antibiotic treatment to reduce the risk of recurrent infection includes the use of antibiotic-impregnated bone cement for prosthesis fixation at revision surgery (3) and the intravenous administration of antibiotics during revision surgery. In Musgrave Park Hospital, Belfast, United Kingdom, gentamicin is incorporated into bone cement and the cephalosporin cefamandole (Kefadol) is used for routine antimicrobial prophylaxis. When patients undergoing revision hip surgery are allergic to cefamandole, erythromycin is usually employed prophylactically.

Gentamicin resistance among bacteria isolated from infected hip joints has been reported. In a study of 33 infected hip joints, Weber and Lautenbach (13) noted that 29% of bacteria isolated preoperatively were resistant to gentamicin. Interestingly, following the use of gentamicin-impregnated bone cement, resistance increased to 41% of bacteria isolated postoperatively. In another study of cemented total hip arthroplasty infection caused by coagulase-negative staphylococci (CNS), Hope et al. (5) reported that the use of gentamicin-impregnated cement in the primary arthroplasty was associated with the emergence of gentamicin-resistant CNS in subsequent infection. Of 34 hip implants at revision surgery in which gentamicin-impregnated cement had been used at the previous operation, 30 (88%) later grew at least one strain of gentamicin-resistant CNS. In contrast, of 57 hip implants at revision surgery in which gentamicin was not included in the bone cement, only 9 (16%) later grew gentamicin-resistant CNS. In addition, an earlier study to determine the efficacy of antimicrobial agents in eradicating the normal skin microbiota prior to surgery reported that 18 of 152 patients (12%) had cefamandole-resistant *Staphylococcus epidermidis*, leading the authors to conclude that preoperative antimicrobial prophylaxis with cefamandole would have failed to protect these patients from the *S. epidermidis* which colonized their skin (11). The aim of the present study was, therefore, to determine the susceptibilities of bacteria isolated from revision hip prostheses to the commonly used antimicrobial agents gentamicin, cefamandole, and erythromycin and also to a range of alternative antimicrobial agents.

Twenty-six of 120 implants removed consecutively from patients undergoing revision hip surgery at Musgrave Park Hospital during the 14-month period from March 1996 to May 1997 were diagnosed as infected (12). From these infected implants, 49 clinical isolates were recovered. Review of the hospital notes for 18 patients with culture-positive implants and 52 patients with culture-negative implants revealed that infection prior to revision was suspected in only 8 cases (11%). Implants from 6 of these patients (75%) were subsequently diagnosed as infected in our study. Seven of the implants were infected by a single *Staphylococcus* sp., and a further three were infected by a combination of two *Staphylococcus* spp. The anaerobic bacterium *Propionibacterium acnes* was isolated as the single infecting organism from 12 implants, and a further 4 implants were infected by a combination of *P. acnes* and a gram-positive coccus. The isolates comprised the following: *S. epidermidis*, 17 strains; *Staphylococcus aureus*, 4 strains; *Staphylococcus hominis*, 3 strains; *Staphylococcus capitis*, 2 strains; *Staphylococcus haemolyticus*, 2 strains; *Staphylococcus sciuri*, 1 strain; *Micrococcus* sp., 1 strain; and *P. acnes*, 19 strains.

The following antimicrobial agents were used: gentamicin sulfate, erythromycin, and fusidic acid (Sigma Chemical Co., Poole, Dorset, United Kingdom); cefamandole naftate as Kefadol (Dista Products Ltd., Basingstoke, United Kingdom); cefotaxime as Claforan (Roussel Laboratories Ltd., Uxbridge, United Kingdom); ciprofloxacin as Ciproxin (Bayer plc, Newbury, United Kingdom), and vancomycin as Vancocin (Eli Lilly and Company Ltd., Basingstoke, United Kingdom). MICs were determined by the broth microdilution method (9, 10). Serial twofold dilutions of each antimicrobial were prepared in cation-supplemented Mueller-Hinton broth (MHB with 50 mg of Ca^{2+} and 25 mg of Mg^{2+} per liter; Unipath Ltd., Basingstoke, United Kingdom) within dilution schemes of 0.5 to 1,024 μg/ml (gentamicin, cefamandole, cefotaxime, and erythromy-

* Corresponding author. Mailing address: School of Pharmacy, The Queen's University of Belfast, 97 Lisburn Rd., Belfast BT9 7BL, United Kingdom. Phone: 01232-272017. Fax: 01232-247794. E-mail: s.gorman@qub.ac.uk.

TABLE 1. Antimicrobial susceptibilities of bacteria isolated from orthopedic implants

Isolate (no. of strains tested)	Test agent	MIC (μg/ml)			% Susceptible	MBC (μg/ml)		
		Range	50%	90%		Range	50%	90%
All (49)	Gentamicin	<0.5–512	8	64		1–>1,024	32	1,024
	Cefamandole	<0.5–64	1	32		<0.5–>1,024	1	64
	Cefotaxime	<0.5–64	2	16		<0.5–>1,024	64	512
	Erythromycin	<0.5–>1,024	16	>1,024		<0.5–>1,024	256	>1,024
	Vancomycin	0.25–2	1	2		1–64	32	64
	Ciprofloxacin	0.125–2	0.5	1		0.125–64	8	32
	Fusidic acid	<0.125–32	1	8		1–>256	16	>256
Staphylococcus spp. (30)	Gentamicin	<0.5–512	16	128	26	1–>1,024	32	1,024
	Cefamandole	<0.5–64	2	64	63	1–512	16	128
	Cefotaxime	<0.5–32	4	16	77	4–>1,024	128	1,024
	Erythromycin	<0.5–>1,024	256	>1,024	6	2–>1,024	>1,024	>1,024
	Vancomycin	0.25–2	1	2	100	1–64	32	64
	Ciprofloxacin	0.125–2	0.5	1	100	0.125–64	16	32
	Fusidic acid	<0.125–16	0.25	16	NA[a]	1–>256	64	>256
P. acnes (19)	Gentamicin	<0.5–16	4	8	NA	2–128	8	64
	Cefamandole	<0.5	<0.5	<0.5	100	<0.5–4	<0.5	1
	Cefotaxime	<0.5–1	<0.5	<0.5	100	<0.5–128	<0.5	2
	Erythromycin	<0.5–>1,024	<0.5	>1,024	NA	<0.5–>1,024	<0.5	>1,024
	Vancomycin	<0.125–1	0.5	0.5	NA	4–>256	8	32
	Ciprofloxacin	0.5–1	1	1	NA	1–128	8	32
	Fusidic acid	<0.125–8	1	2	NA	2–>256	16	32

[a] NA, no MIC breakpoint approved by the National Committee for Clinical Laboratory Standards.

cin) and 0.125 to 256 μg/ml (vancomycin, ciprofloxacin, and fusidic acid). The microdilution trays were stored in sealed plastic bags at −70°C and used within 3 weeks.

The inoculum for facultative isolates to be tested was prepared by adjusting the turbidity of an actively growing broth culture in MHB to an optical density at 540 nm equivalent to 1×10^8 CFU/ml. The suspension was further diluted to provide a final inoculum density of 5×10^5 CFU/ml. Anaerobic isolates to be tested were grown on anaerobic horse blood agar plates at 37°C for 48 h in an anaerobic chamber (Don Whitley Scientific, Shipley, United Kingdom). The inoculum was prepared by suspending bacteria from these plates in prereduced MHB, which provided optimal growth conditions for the *P. acnes* isolates. The suspension was then adjusted by spectrophotometric measurement to provide a final inoculum density of 10^6 CFU/ml.

The microdilution trays were removed from the freezer and thawed, and trays to be used for anaerobic bacteria were equilibrated in the anaerobic chamber for 4 h. The final inoculum (100 μl) was added to each well of the microdilution trays. Facultative isolates were incubated in air at 37°C for 24 h, and the anaerobic *P. acnes* isolates were incubated in the anaerobic chamber at 37°C for 48 h. After incubation, the MIC was read as the lowest concentration of each antimicrobial agent which inhibited visible growth of the test isolate. Quality assurance testing was performed with *Enterococcus faecalis* ATCC 22697 and *Bacteroides fragilis* ATCC 25285.

In order to determine the minimum bactericidal concentration (MBC), 20-μl aliquots were inoculated onto Mueller-Hinton agar plates which were incubated as described previously. The MBC was defined as the lowest antibiotic concentration that produced greater than 99.9% killing of the initial inoculum.

The results of this study are summarized in Tables 1 and 2. Control strains gave reproducible results, with MICs within National Committee for Clinical Laboratory Standards limits and 1 dilution of the mean. The majority of facultative isolates were resistant to gentamicin and erythromycin. In contrast, there was less resistance of facultative isolates to cefamandole, cefotaxime, and fusidic acid. Vancomycin and ciprofloxacin were most effective against the facultative isolates. All *P. acnes* strains were susceptible to cefamandole, cefotaxime, vancomycin, ciprofloxacin, and fusidic acid. However, higher concentrations of both gentamicin and erythromycin were required to inhibit the *P. acnes* strains. Based on overall MBCs at which 90% of strains tested were killed, ciprofloxacin was the most active bactericidal agent tested, followed in decreasing order by cefamandole, vancomycin, cefotaxime, gentamicin, fusidic acid, and erythromycin.

Although higher antibiotic concentrations are achieved locally with antibiotic-impregnated bone cement (4), this in vitro study has shown by the high numbers of gentamicin-resistant bacteria which were isolated that the routine use of gentamicin-impregnated bone cement may be ineffective. This finding was not unexpected as virtually all the retrieved implants had been fixed in place with gentamicin-impregnated bone cement, and it supports the results previously reported by Weber and Lautenbach (13). The use of erythromycin peroperatively in patients who are allergic to cephalosporins may also be ineffective, based on the high proportion of erythromycin-resistant bacteria isolated. The results described herein suggest that the use of other agents, for example, vancomycin and ciprofloxacin, in bone cement and peroperatively, respectively, could be more effective for the elimination of implant infection at the time of revision hip surgery and for the prevention of further implant infection. Previous studies have reported that the stability and physicochemical properties of vancomycin are not adversely affected by its addition to bone cement (7) and have also shown that the drug is released in sufficient concentrations to treat and prevent experimentally induced *S. aureus* osteomyelitis in rats (2). Further work to determine the efficacy of these antibiotics against bacteria growing within adherent biofilms on the surface of implant biomaterials is under way.

TABLE 2. Antimicrobial susceptibilities of staphylococcal species isolated from orthopedic implants

Isolate (no. of strains tested)	Test agent	MIC (μg/ml)			MBC (μg/ml)		
		Range	50%	90%	Range	50%	90%
S. epidermidis (17)	Gentamicin	<0.5–512	16	256	1–>1,024	128	>1,024
	Cefamandole	<0.5–64	4	32	1–512	16	64
	Cefotaxime	<0.5–32	4	16	4–>1,024	128	512
	Erythromycin	<0.5–>1,024	>1,024	>1,024	2–>1,024	>1,024	>1,024
	Vancomycin	1–2	2	2	8–64	16	64
	Ciprofloxacin	0.25–1	0.5	1	0.5–64	16	32
	Fusidic acid	<0.125–16	0.5	16	1–>256	>256	>256
S. aureus (4)	Gentamicin	16–32			16–32		
	Cefamandole	32			64–512		
	Cefotaxime	2–4			256–>1,024		
	Erythromycin	2–16			2–>1,024		
	Vancomycin	0.5–1			16–32		
	Ciprofloxacin	0.5–1			1–32		
	Fusidic acid	<0.125–0.25			16–128		
S. hominis (3)	Gentamicin	<0.5–32			2–64		
	Cefamandole	1–64			1–>1,024		
	Cefotaxime	2–4			64–>1,024		
	Erythromycin	128–>1,024			1,024–>1,024		
	Vancomycin	1			16–32		
	Ciprofloxacin	0.25–2			0.25–32		
	Fusidic acid	0.25–32			4–>256		
S. capitis (2)	Gentamicin	<0.5–16			8–64		
	Cefamandole	1			1–16		
	Cefotaxime	2–4			128–512		
	Erythromycin	256			256–>1,024		
	Vancomycin	1			32		
	Ciprofloxacin	0.25			8–16		
	Fusidic acid	<0.125–0.25			32–64		
S. haemolyticus (2)	Gentamicin	64			256–512		
	Cefamandole	32–64			64		
	Cefotaxime	32–64			64–256		
	Erythromycin	32–>1,024			>1,024		
	Vancomycin	1–2			1–32		
	Ciprofloxacin	0.25			8–16		
	Fusidic acid	1–8			4–>256		
S. sciuri (1)	Gentamicin	4			16		
	Cefamandole	1			1		
	Cefotaxime	4			64		
	Erythromycin	64			>1,024		
	Vancomycin	1			32		
	Ciprofloxacin	0.5			32		
	Fusidic acid	0.25			>256		
Micrococcus sp. (1)	Gentamicin	16			32		
	Cefamandole	1			8		
	Cefotaxime	2			256		
	Erythromycin	>1,024			>1,024		
	Vancomycin	0.25			1		
	Ciprofloxacin	2			2		
	Fusidic acid	<0.125			4		

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Detection of Prosthetic Hip Infection at Revision Arthroplasty by Immunofluorescence Microscopy and PCR Amplification of the Bacterial 16S rRNA Gene

MICHAEL M. TUNNEY,[1] SHEILA PATRICK,[1]* MARTIN D. CURRAN,[3] GORDON RAMAGE,[1] DONNA HANNA,[1] JAMES R. NIXON,[4] SEAN P. GORMAN,[2] RICHARD I. DAVIS,[5] AND NEIL ANDERSON[5]

Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, Belfast BT12 6BN,[1] Regional Histocompatibility and Immunogenetics Laboratory, Belfast City Hospital, Belfast BT9 7TS,[3] Withers Orthopaedic Centre, Musgrave Park Hospital, Belfast BT9 7JB,[4] School of Pharmacy, The Queen's University of Belfast, Belfast BT9 7BL,[2] and Department of Pathology, The Royal Group of Hospitals and Dental Hospital Health and Social Services Trust, Belfast BT12 6BA,[5] United Kingdom

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In this study the detection rates of bacterial infection of hip prostheses by culture and nonculture methods were compared for 120 patients with total hip revision surgery. By use of strict anaerobic bacteriological practice during the processing of samples and without enrichment, the incidence of infection by culture of material dislodged from retrieved prostheses after ultrasonication (sonicate) was 22%. Bacteria were observed by immunofluorescence microscopy in 63% of sonicate samples with a monoclonal antibody specific for *Propionibacterium acnes* and polyclonal antiserum specific for *Staphylococcus* spp. The bacteria were present either as single cells or in aggregates of up to 300 bacterial cells. These aggregates were not observed without sonication to dislodge the biofilm. Bacteria were observed in all of the culture-positive samples, and in some cases in which only one type of bacterium was identified by culture, both coccoid and coryneform bacteria were observed by immunofluorescence microscopy. Bacteria from skin-flake contamination were readily distinguishable from infecting bacteria by immunofluorescence microscopy. Examination of skin scrapings did not reveal large aggregates of bacteria but did reveal skin cells. These were not observed in the sonicates. Bacterial DNA was detected in 72% of sonicate samples by PCR amplification of a region of the bacterial 16S rRNA gene with universal primers. All of the culture-positive samples were also positive for bacterial DNA. Evidence of high-level infiltration either of neutrophils or of lymphocytes or macrophages into associated tissue was observed in 73% of patients. Our results indicate that the incidence of prosthetic joint infection is grossly underestimated by current culture detection methods. It is therefore imperative that current clinical practice with regard to the detection and subsequent treatment of prosthetic joint infection be reassessed in the light of these results.

Over 50,000 total hip replacement operations are performed annually in the United Kingdom and about 200,000 are performed annually in the United States. The majority of patients undergoing hip replacement experience dramatic relief of pain and restoration of satisfactory hip function (15). A proportion (approximately 20% in Europe [6]) fail, and prosthesis removal and replacement are usually required, with attendant patient trauma and increased medical costs (9). Aseptic mechanical loosening is reported to be the most common cause of prosthetic joint failure (15), after which standard bacteriological culture of specimens from periprosthetic tissue or aspirates is used to detect infection. Estimates of the incidence of infection as a cause of hip failure are usually in the range of 10% (12) to 15% (20), although some studies report an incidence as low as 2% (3) of all revision operations. Failure of the second implant postrevision, however, may be due to infection in up to 40% of patients (10). It has been suggested that the higher rate of infection postrevision could be due to a longer operating time, increased scar tissue formation, or unrecognized infection at the initial revision operation. Our recent study at The Queen's University of Belfast (37) implicates unrecognized infection as a major cause of prosthetic joint failure. In this study, which was the first to combine sampling by mild ultrasonication to dislodge the bacteria growing within adherent biofilms on the surface of the removed prosthesis with the use of strict anaerobic techniques, we cultured bacteria from 22% of retrieved prostheses (26 of 120 implants). Detection of infections which arise from opportunistic pathogens of the normal microbiota, in particular, the skin, is always problematic, as a clear distinction must be made between infection and potential contamination. This is particularly the case when broth enrichments are used. Our prosthetic joint isolates were obtained by direct plating of the dislodged material onto agar plates and not as a result of broth enrichment. Also, ultrasonication to dislodge the biofilm was required to detect infection by culture. Furthermore, mock processing of a number of autoclaved implants failed to detect any bacteria. It is therefore highly unlikely that the bacteria isolated were present in the sample as a result of exogenous contamination.

Review of the notes for 18 of the 26 patients with culture-positive implants revealed that infection was suspected as the cause of loosening in only 6 patients. Joint fluid, aspirated preoperatively and prior to antibiotic administration, and tis-

* Corresponding author. Mailing address: Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast, Grosvenor Rd., Belfast BT12 6BN, United Kingdom. Phone: 01232-263205. Fax: 01232-439181. E-mail: s.patrick@qub.ac.uk.

sue removed at the time of surgery were processed by the routine clinical laboratory. In only two patients was infection confirmed and were bacteria cultured. In our laboratory, viable bacteria were isolated from only five tissue samples corresponding to prostheses from which bacteria were isolated. This lack of bacteria in cultures of the tissue samples may be due to either the bacteriostatic or the bactericidal effects of cefamandole administered at the time of operation or the host's defenses. In contrast, bacteria growing within a biofilm on device surfaces have greater resistance to antibiotics (40). Investigation of the antibiotic sensitivity of our isolates indicated that the majority were more than 1,000 times more resistant (minimum bactericidal concentration, >1,024 μg/ml) to cefamandole when growing within in vitro model biofilms than when growing in broth culture (39, 40). These results suggest that the device materials are the major sites colonized by the bacteria in this type of infection and highlight the importance of sampling directly from the prosthesis. In our study the anaerobic bacterium *Propionibacterium acnes* was isolated either alone or in association with *Staphylococcus* spp. from 62% of patients. Other published studies report either very low isolation rates for anaerobic bacteria (2) or failure to isolate any (1, 13). This indicates that adherence to strict anaerobic bacteriological practice can increase the detection of prosthetic joint infection and alter our perception of the major causative organisms.

When pathological examination of associated tissue has been carried out, a good correlation between detection of infection by culture and the presence of neutrophilic polymorphonuclear leukocytes in the tissue has been obtained (1, 12, 13, 28). For example, a recent study which involved the detection of infection by both culture and histological assessment of a tissue inflammatory response in five or six tissue specimens reported that 41 of 297 (14%) patients were infected (2). It has been suggested that the observation of neutrophilic infiltration in the associated tissues is a useful means of diagnosing prosthetic joint infection.

Our studies of the inflammatory response in associated tissues showed that in eight of the culture-positive patients neutrophils were not detected, although lymphocytes or macrophages were present. It may be that the lymphocyte or macrophage infiltration is in response to the slow release of bacterial components from a bacterial biofilm growing on the device surface. Histopathological examination of associated tissue samples taken from culture-negative patients showed that 8 had evidence of a high level of neutrophilic infiltration and that a further 36 with low levels or no evidence of neutrophils had large numbers of lymphocytes or macrophages. In total, 87% of our culture-negative patients had evidence either of neutrophilic or of lymphocyte or macrophage infiltration into tissue and may have been infected. These results suggest that even with improved sampling procedures the detection of infection by culture may still be an underestimate of the real incidence of infection. Our study highlights potential inadequacies in current clinical diagnostic methodology (37).

The detection of bacterial rRNA genes as an indicator of the presence of bacteria is an established technique that has been used for the detection of both environmental and medically important bacteria (17, 42). PCR amplification of bacterial 16S rRNA genes has been used successfully to detect bacteria that cause a variety of infections including postoperative endophthalmitis (19, 24), septic arthritis (5), and meningitis (30). In infections such as those of prosthetic joints, however, in which the infectious agents are opportunistic pathogens that may be members of the normal microbiota (e.g., the skin), the potential contamination of samples must be addressed. The direct immunological detection of bacteria in clinical samples can be achieved by the use of monoclonal antibodies (MAbs) and polyclonal antiserum prepared against the bacteria implicated in the clinical infection (32). This technique allows the direct visualization of the bacterial morphology and visual comparison with potential skin-flake contamination.

The aim of the present study was, first, to determine if prosthetic hip infection could be detected reliably by nonculture methods with culture-positive samples. Second, in the light of the inflammatory response in the associated tissues, the aim of the study was to ascertain if nonculturable bacteria were present in culture-negative patient samples. We therefore compared the use of (i) immunolabelling in conjunction with fluorescence microscopy and (ii) PCR amplification of a region of the bacterial 16S rRNA gene for the detection of prosthetic hip infection while taking suitable steps to eliminate and to control for potential contamination by bacteria of the normal microbiota.

MATERIALS AND METHODS

Clinical sample collection and processing. One hundred twenty prosthetic hip implants were retrieved from patients undergoing revision hip surgery at Musgrave Park Hospital, Belfast, United Kingdom, during the 14-month period from March 1996 to April 1997. All patients underwent standardized preoperative hygiene procedures. Following skin preparation with Betadine (Seton Healthcare, Oldham, United Kingdom) the incision area was covered with an adhesive plastic drape. All operations were carried out in operating theaters in which a vertical laminar air flow provided a clean environment and all members of the operating team wore disposable impervious drapes. Routine antibiotic prophylaxis consisted of 2 g of cefamandole (Kefadol; Dista Products Ltd., Basingstoke, United Kingdom) given intravenously at the time of anesthetic induction. Further 1-g doses of cefamandole were given 8 and 16 h after surgery. Cefamandole is recommended by the British National Formulary for surgical prophylaxis. It is a broad-spectrum antibiotic active against both gram-positive and gram-negative bacteria. On removal, the surgeon aseptically placed the femoral and acetabular components in separate sterile bags. Tissue in contact with the implants was also removed and placed in sterile bottles. The femoral and acetabular components and the tissue samples were immediately placed in an anaerobic jar for transportation to an anaerobic cabinet (37).

Bacterial isolation and identification. Samples were handled within the closed atmosphere of an anaerobic cabinet (Don Whitley Mk. III anaerobic cabinet; 80% N_2, 10% CO_2, and 10% H_2; Don Whitley Scientific Ltd., Shipley, United Kingdom). The gas atmosphere of the cabinet was continuously pumped through a solution of 2% glutaraldehyde, and operators wore surgical gloves throughout processing. Bacteria growing within adherent biofilms on the surface of the prostheses were dislodged by mild ultrasonication (5 min, 50 Hz) into Ringer's solution (25% [vol/vol]) containing cysteine (0.05% [vol/vol]) as a reducing agent (37). This procedure has been shown to have no effect on the viability of pure culture isolates. The use of Ringer's solution rather than broth as a diluent ensured that the bacteria did not multiply within the sonicate prior to further processing. Determination of total viable bacterial counts was performed as follows: Volumes (0.5 ml) of sonicate were plated onto each of five blood agar (BA) and five anaerobic blood agar (ABA) plates. A known weight of tissue was homogenized (3 min) in Ringer's solution (25% [vol/vol], 5 ml). Three 0.5-ml volumes of homogenized tissue were plated onto BA and ABA plates. The remaining volume of homogenized tissue was added in equal amounts to tryptone soy broth (TSB) and cooked meat broth (CMB) for enrichment. BA and ABA plates were incubated at 37°C aerobically and anaerobically, respectively, and were examined after 1, 2, 4, and 7 days. Samples were recorded as positive if colonies were observed on a minimum of four of the five plates. The TSB and CMB were incubated at 37°C aerobically and anaerobically, respectively. Both broths were subcultured on days 7 and 14: on BA for aerobic incubation and on ABA for anaerobic incubation. All plates were incubated at 37°C for 48 h. To determine the likelihood that contaminating bacteria were introduced during the sampling procedure, 20 retrieved orthopedic implants were sterilized in a hot-air oven and were processed as described above. In addition, three researchers in our laboratory scraped flakes from the surfaces of their skin into sterile bags containing Ringer's solution, which were then processed as described above.

Pure bacterial cultures, obtained by picking isolated colonies from the viable count plates, were identified with commercially available kits. Cultures were stored both on Protect Bacterial Preservers (Technical Service Consultants Ltd., Heywood, United Kingdom) at −70°C and on Mueller-Hinton agar slopes at 4°C, which were subcultured at 3-month intervals. The remaining sonicate from each sample was centrifuged (1,000 × *g*, 20 min), and the concentrated sonicate was stored in 1-ml aliquots at −70°C for further analysis.

Bacterial strains. The strains used in this study were *Bacteroides fragilis* NCTC 9343 (National Collection of Type Cultures, Colindale, United Kingdom); *Peptostreptococcus magnus* NCTC 11804; *Peptostreptococcus micros* NCTC 11808;

Escherichia coli O128, kindly supplied by C. Smyth, Trinity College Dublin, Ireland; and *Corynebacterium diphtheriae*, *Corynebacterium hofmanni*, and *Corynebacterium xerosis* from the culture collection of the Department of Microbiology and Immunobiology, School of Clinical Medicine, The Queen's University of Belfast. The clinical isolates used in this study (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus capitis*, *Propionibacterium acnes*, and *Micrococcus* sp.) were all isolated at the Department of Microbiology and Immunobiology, School of Medicine, from retrieved prosthetic hip implants as described above (37).

Immunological detection of bacteria. (i) Production of polyclonal antisera. A New Zealand White rabbit was immunized with whole cells of *S. epidermidis*. The rabbit was inoculated subcutaneously at four sites on the back with 0.1 ml of a bacterial suspension of 10^8 CFU/ml in 0.01 M phosphate-buffered saline (PBS [pH 7.4]; 0.15 M NaCl, 0.0075 M Na_2HPO_4, 0.0025 M $NaH_2PO4 \cdot 2H_2O$). Two further inoculations of bacteria in PBS were made at approximately monthly intervals, and the rabbit was test bled 2 weeks after the final inoculation. The reactivity of the antiserum was then tested by immunofluorescence microscopy (IFM) as described previously (23).

(ii) Production of MAbs. Four BALB/c mice were immunized with whole cells of two *P. acnes* strains. The mice were inoculated intraperitoneally with 0.2 ml of a bacterial suspension of 10^8 CFU/ml in 0.01 M PBS. Further 0.2-ml inoculations were given after 1 month. Serum was tested by immunofluorescence after 3 days, and the mice with the highest titer of antiserum were inoculated again 1 week later. One mouse was killed after 5 days, and the spleen cells from the mouse were fused with P3X 63 Ag8-653 (NS-0/1) mouse myeloma cells by a modification of the method of Galfre and Milstein (16) as described previously (23). Hybridoma cell lines, which produced *P. acnes*-specific antibodies, were then cloned by limiting dilution (18).

(iii) IFM. A modification of the IFM procedure described by Lutton et al. (23) was used. Samples of sonicate obtained from prosthetic joints (1 ml) were further centrifuged (10,000 × *g*; 20 min), and the resulting pellets were resuspended in 100 μl of PBS. Samples (10 μl) were then applied in duplicate to multiwell slides. The slides were air dried and then fixed in 100% methanol for 10 min at −20°C.

The slides were dually labelled for IFM with a combination of MAb QUBPa3 supernatant, which reacted with all *P. acnes* strains isolated in our previous study, and *S. epidermidis* polyclonal antiserum, which reacted with all *Staphylococcus* spp. strains isolated in our previous study (37). The reactivity of MAb QUBPa3 with *S. aureus*, *S. epidermidis*, *B. fragilis*, *E. coli*, *P. magnus*, *P. micros*, *C. diphtheriae*, *C. hofmanni*, and *C. xerosis* was also tested. Similarly, the reactivity of the *S. epidermidis* polyclonal antiserum with *B. fragilis*, *E. coli*, and the gram-positive anaerobic cocci *P. magnus* and *P. micros* was tested. For dual labelling, the slides were incubated with undiluted MAb QUBPa3 supernatant, washed, and incubated with rabbit anti-*S. epidermidis* polyclonal antiserum diluted 1 in 200 in PBS. The slides were again washed and then incubated simultaneously with sheep anti-rabbit fluorescein conjugate (Sigma) and goat anti-mouse rhodamine conjugate (Sigma). After a final wash, all slides were mounted with glycerol-PBS containing an antiphotobleaching agent (Citifluor; Agar Scientific Ltd, Essex, United Kingdom) and were examined with a Leitz fluorescent microscope.

For clinical sonicate samples the detection of bacteria on duplicate wells by immunofluorescence was given a score of between 0 and 3 by using the following criteria: 0, no bacteria; 1, 1 to 10 bacteria/well; 2, 10 to 50 bacteria/well; 3, 50 or more bacteria/well.

To determine whether bacteria detected by immunofluorescence in concentrated sonicate samples could have resulted from skin-flake contamination, skin from researchers working at the Department of Microbiology and Immunobiology, School of Clinical Medicine, was scraped with sterile scalpels into 1-ml volumes of PBS. These were then processed in the same way as the sonicate samples and were examined by dual-labelling IFM.

(iv) CSLM. Selected wells were examined by confocal scanning laser microscopy (CSLM) with a Leica NCS-NT confocal scanning laser microscope.

Molecular detection of bacteria. (i) PCR sensitivity. Sensitivity tests were performed with *E. coli*, *B. fragilis*, and all the organisms isolated from retrieved orthopedic implants and listed above. Facultative isolates were grown on BA at 37°C for 24 h, and anaerobic isolates were grown on ABA in the anaerobic cabinet for a similar time period. Single colonies of each isolate were inoculated into TSB (10 ml); *B. fragilis*, however, was inoculated into defined minimal medium (41). Isolates were incubated as described above, and the actively growing cultures were adjusted to an optical density at 540 nm equivalent to 10^8 CFU/ml and were diluted in 10-fold serial dilutions with PBS. Viable cells were counted as the number of CFU by triplicate plating of diluted samples on either BA or ABA and counting the colonies after incubation at 37°C for 24 h. From 10-fold serial dilutions containing from 10^8 to 10^0 CFU/ml, 1-ml volumes were retained for DNA extraction.

(ii) PCR laboratory conditions and control measures. PCR was carried out under stringent conditions in a hospital diagnostic laboratory accredited by the American Society for Histocompatibility and Immunogenetics. All DNA manipulations pre- and post-PCR were performed in separate designated rooms with separate pipetting devices to avoid contamination of the samples with foreign DNA. Furthermore, as in other studies (4, 27), UV light was used to irradiate all equipment used in the preamplification steps to prevent contaminating DNA from causing false-positive results. Master-mixture water controls and DNA extraction controls were used for every batch of samples processed.

TABLE 1. Comparison of the detection rates of prosthetic hip infection by different methods

Method of detection	No. of samples	No. of positive samples	% Positive samples
Culture of tissue only	120	5	4
Culture of tissue and implants[a]	120	26	22
Immunofluorescence microscopy	113	71	63
16S rRNA gene amplification	118	85	72
Inflammatory cell infiltration	81	59[b]	73

[a] Use of mild ultrasonication to dislodge bacteria growing within adherent biofilms and strict anaerobic procedures.
[b] Inflammatory score greater than 1.

(iii) DNA extraction. Aliquots (1 ml) of prosthesis sonicate or bacterial culture were centrifuged at 10,000 × *g* for 15 min. The pellets were resuspended by vortex mixing in 200 μl of cell lysis buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% sodium dodecyl sulfate, proteinase K [100 μg/ml]). This reaction took place at 55°C for 3 h, after which the temperature was adjusted to 37°C and the reaction mixture was incubated overnight. The temperature of the samples was increased to 55°C for 1 h before DNA extraction was performed by the addition of equal volumes of saturated phenol-chloroform. After vortex mixing and centrifugation at 10,000 × *g* for 15 min, the aqueous phase was removed and the DNA was precipitated by the addition of 100% ethanol (2.5 volumes), 3 M sodium acetate (pH 5.2) (0.1 volume), and 2 μl of See DNA (Amersham, Aylesbury, United Kingdom). Following brief vortex mixing and centrifugation at 10,000 × *g* for 15 min, the supernatant was poured off and the DNA pellet was washed by the addition of 1 ml of 70% ethanol. Following further brief vortex mixing and centrifugation at 10,000 × *g* for 15 min, any residual ethanol traces were removed by vacuum drying. The extracted DNA was then dissolved in 50 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and was stored at −20°C.

(iv) Oligonucleotide primers. Oligonucleotide primers were synthesized by Perkin-Elmer-Applied Biosystems, Warrington, United Kingdom. The target DNA sequence was the 16S rRNA gene. Sequence alignment by the clustal method with a weighted residue weight table (34) was carried out with the 16S rRNA genes of the following bacteria: *S. capitis*, *S. epidermidis*, *Staphylococcus haemolyticus*, *P. acnes*, *Micrococcus agilis*, *B. fragilis*, *E. coli*, and a *Peptostreptococcus* sp. The primer set selected was D1 (5′-GAG GAA GGT RGG GAY GAC GT) and D2 (5′-AGG CCC GGG AAC GYA TTY ACC G) for amplification of a 216-bp fragment of the 16S rRNA gene. The positions of D1 and D2 are 1199 to 1219 and 1394 to 1415 of the *E. coli* 16S rRNA positions, respectively (R = AG, Y = CT).

(v) DNA amplification. The PCR mixture, which was made up to 50 μl in sterile double-distilled water, contained 5 μl of 10× PCR buffer (Perkin-Elmer-Applied Biosystems), 5 μl of $MgCl_2$ (25 mM), each deoxynucleotide triphosphate (Pharmacia Biotech, Milton Keynes, United Kingdom) at a concentration of 200 μM, 20 pM each primer, and 3 U of Ampli*Taq* polymerase (Perkin-Elmer-Applied Biosystems). Two microliters of lysate containing target DNA was added to the PCR mixture, which was incubated at 96°C for 5 min. PCR was performed for 30 cycles of 1 min at 96°C, 2 min at 55°C, and 1 min at 72°C using a Perkin-Elmer Gene-Amp PCR System 9600 (Perkin-Elmer-Applied Biosystems). The final cycle ended with a 5-min extension at 72°C, and the PCR products were stored in the thermocycler at 15°C until they were collected.

After amplification, 6 μl of the amplified product was run on a 1.5% agarose gel in 1× Tris-borate-EDTA. DNA bands were detected by ethidium bromide staining and were visualized by UV light photography.

RESULTS

Incidence of infection. Our results indicate that the use of nonculture methods as opposed to culture methods significantly ($P < 0.05$; chi-square test) increases the level of detection of infected prostheses (Table 1). By including only samples positive by both IFM and 16S rRNA detection and with an inflammatory cell infiltration score (see Table 3) of greater than 1, a conservative estimate for the level of infection in culture-negative samples is 25 of 94 (27%). In contrast, if all samples that were positive by either IFM or 16S rRNA detection are included, the level of infection increases to 69 of 94 (73%) samples.

Immunological detection of bacteria. MAb QUBPa3, which reacted with all of the *P. acnes* strains isolated in our previous study (37), did not cross-react with *S. aureus*, *S. epidermidis*, *B. fragilis*, *E. coli*, *P. magnus*, *P. micros*, *C. diphtheriae*, *C. hof-*

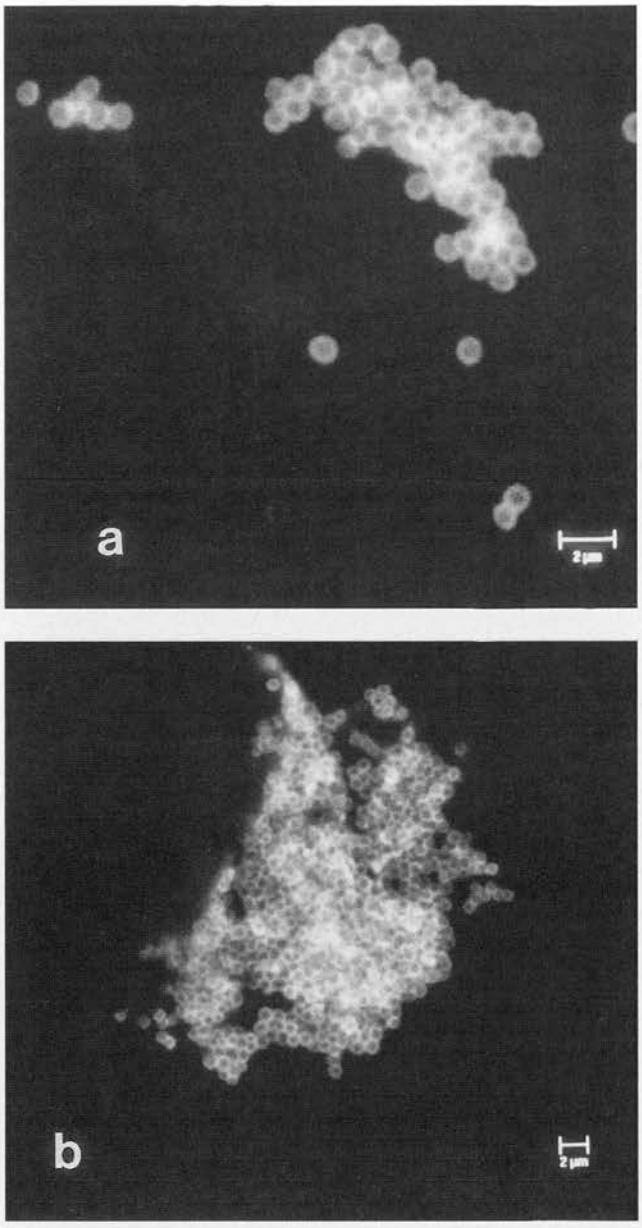

FIG. 1. Examples of confocal laser scanning micrographs of bacteria in material removed by ultrasonication (sonicate) from culture-negative hip prostheses to illustrate coccoid cells present singly and in small groups (a) and in a large aggregate (b). Bacteria were labelled with anti-*Staphylococcus* spp. polyclonal antiserum and an anti-*P. acnes*-specific MAb, followed by labelling with suitable fluorescently conjugated secondary antibodies.

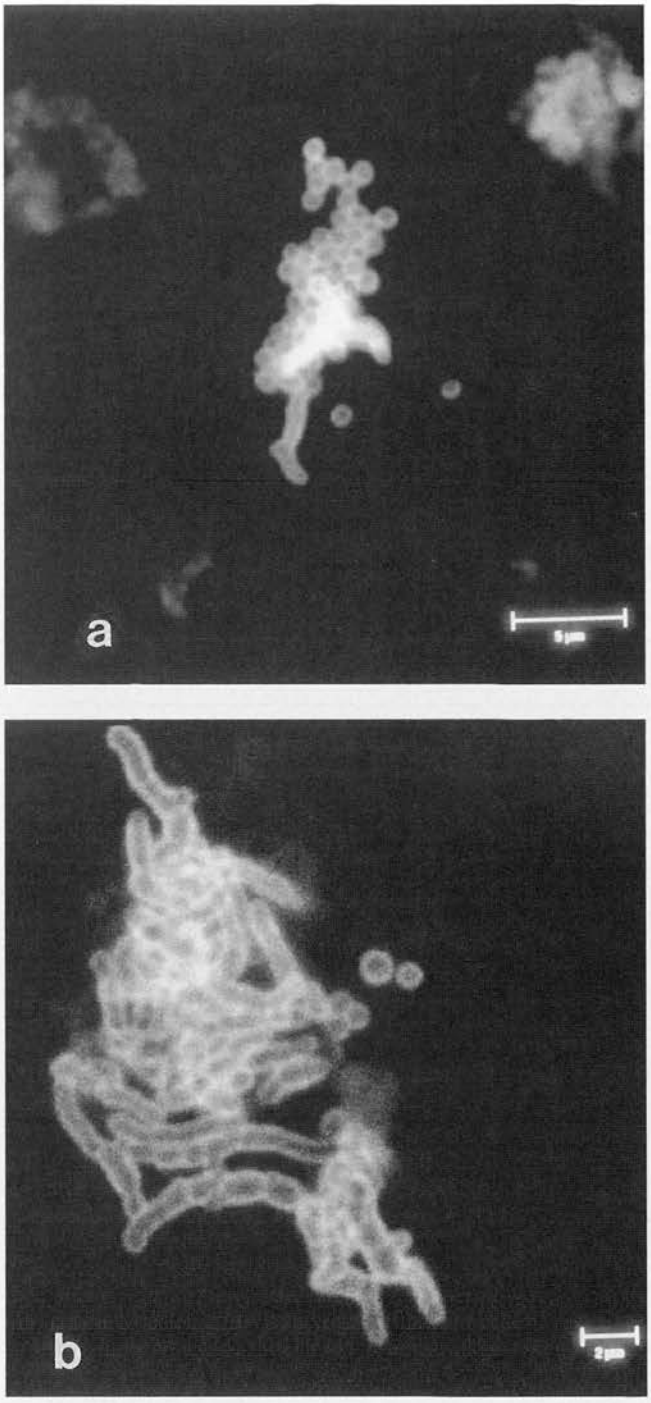

FIG. 2. Confocal laser scanning micrographs of sonicates obtained from culture-negative hip prostheses to illustrate a large number of coccoid cells associated with a smaller number of coryneform cells (a) and a large number of coryneform cells associated with a smaller number of coccoid cells (b) labelled as described in the legend to Fig. 1.

manni, or *C. xerosis* by IFM. Similarly, the polyclonal antiserum raised to *S. epidermidis*, which reacted with all *Staphylococcus* strains isolated in our previous study (37), did not cross-react with *B. fragilis*, *E. coli*, *P. magnus*, or *P. micros*.

Bacteria were observed singly, in small groups, and in large aggregates by IFM (Fig. 1a and b). In addition, both *P. acnes* and *Staphylococcus* spp. were found together in large aggregates in several instances (Fig. 2a and b). The aggregates were between 3 and 4.5 μm in depth, as estimated by CSLM, and consisted of several layers of bacterial cells (Fig. 3). These aggregates were observed only after ultrasonication of the prostheses. When a selected number of prostheses were placed in Ringer's solution and the solution was removed and processed without ultrasonication, the occasional individual bacterial cell was observed in samples in which large aggregates were observed after ultrasonication. No bacteria were visible by IFM when three researchers at the Department of Microbiology and Immunobiology, School of Clinical Medicine,

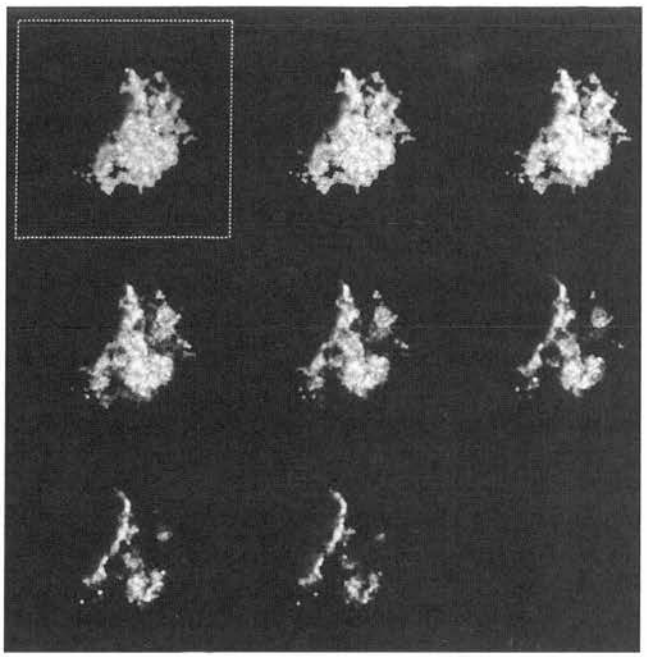

FIG. 3. Confocal laser scanning micrograph illustrating the depth of the dislodged biofilm aggregate of coccoid cells shown in Fig. 1b. The series of images were captured at 0.5-μm intervals from the top (image in the box) to the bottom (images displayed left to right) of the aggregate. The depth of this aggregate was estimated to be 3.5 μm.

scraped their skin flakes into the bags containing Ringer's solution that are normally used for the retrieved prostheses, and the contents of the bags were then processed in the same manner as bags containing prostheses. Concentrated skin scrapings were also applied to microscope slides and were processed for IFM. No large aggregates of bacteria which resembled those observed in the sonicates were detected. IFM revealed sparse small groups of bacteria (up to five or six cells), some isolated bacterial cells, and skin cells (Fig. 4). Skin cells were not observed in sonicates.

All 24 clinical sonicate samples which were positive by culture were also positive by IFM (Table 2). In 11 of the samples the same bacteria that had been isolated by culture were detected by IFM. A further 11 samples that were positive by culture for either a *Staphylococcus* sp. or *P. acnes* were positive for both organisms by IFM.

Bacteria were also detected by IFM in 47 of the 89 (53%) culture-negative samples examined. *Staphylococcus* spp. and *P. acnes* were detected alone in 20 and 19 samples, respectively, and both organisms were detected in a further 8 samples (Table 3). *Staphylococcus* spp. were found in greater quantity than *P. acnes*, with 17 of the 20 *Staphylococcus*-positive samples having IFM scores of 2 or 3, which corresponds to between 10 and greater than 50 bacteria per field of view. In comparison, only 3 of 19 *P. acnes*-positive samples had a similar score.

Molecular detection of bacteria. The lower limits of detection of *S. aureus*, *S. epidermidis*, *S. hominis*, *S. capitis*, *P. acnes*, *Micrococcus* spp., *E. coli*, and *B. fragilis* bacterial cells by PCR amplification of a region of the bacterial 16S rRNA gene were examined. Results indicated that, by our protocol, *P. acnes* and *Micrococcus* spp. were detected only at concentrations greater than or equal to 10^5 CFU/ml (data not shown). The remaining six organisms could be detected if they were present in numbers greater than or equal to 10^4 CFU/ml (data not shown).

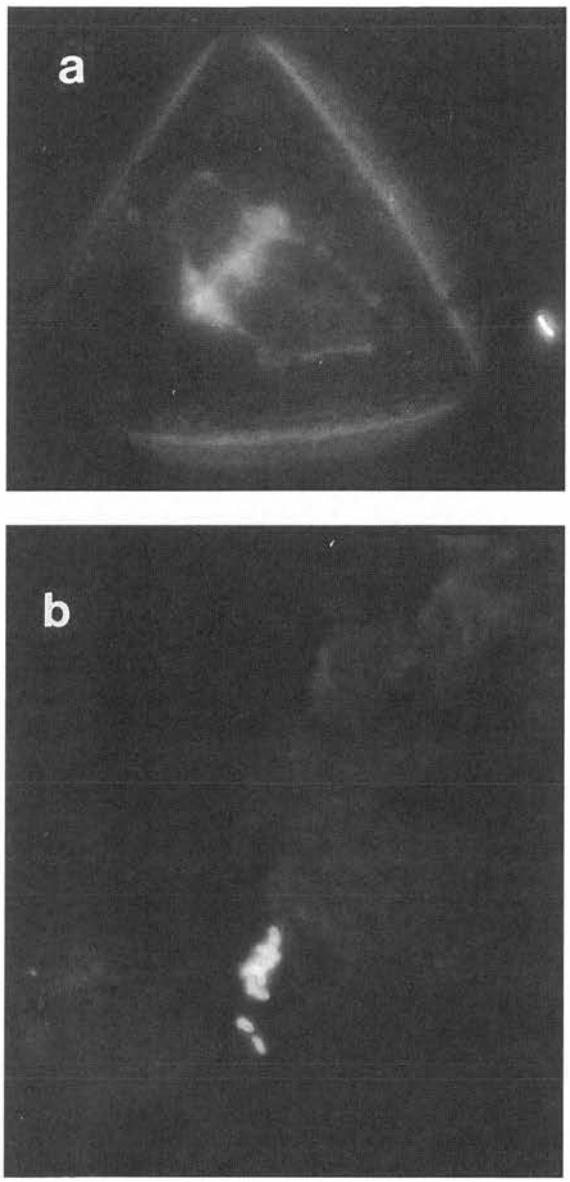

FIG. 4. Immunofluorescence micrographs of material scraped from skin illustrate an isolated bacterial cell and a skin cell (a) and a small group of bacteria (b). Immunolabelling was carried out as described in the legend to Fig. 1.

Bacterial DNA was amplified from sonicate samples of all 25 implants which were culture positive (Table 2). Bacterial DNA was also amplified from sonicate samples of 60 of the 93 (65%) culture-negative implants examined (Table 3).

Comparison of tissue pathology and immunological and molecular detection. All 25 of the culture-positive samples were also positive by IFM and 16S rRNA gene amplification. Tissue pathology results were available for 18 of these patients, and in 8 of these patients neutrophilic infiltration was not observed. Lymphocytes or macrophages were, however, observed in all 18 patients (Table 2). A giant cell-foreign body reaction was also noted in three of the culture-positive tissue samples.

Of the 94 culture-negative samples, 38 (40%) were positive by both IFM and 16S rRNA gene amplification. Tissue pathology was available for 32 of the 38 samples, and there was

TABLE 2. Bacteria detected from culture-positive retrieved prosthetic hip implants by culture, IFM, and bacterial 16S rRNA gene detection and associated tissue inflammatory response

Sample no.	Bacterium(a) isolated	Tissue infiltration by inflammatory cells (score)[a]			IFM result		16S rRNA gene detection
		PMN[b]	LYM[c]	MAC[d]	Morphology	Score[e]	
1	*S. epidermidis*	1	1	2	Cocci	3	+
2	*S. epidermidis*	2	1	3	Cocci	3	+
3	*S. aureus*	0	1	0	Cocci	2	+
4	*S. capitis*	0	0	1	Cocci	1	+
5	*S. epidermidis*	0	1	2	Cocci and *P. acnes*	2	+
6	*S. hominis*	0	1	2	Cocci and *P. acnes*	1	+
7	*S. epidermidis*	1	1	3	*P. acnes*	2	+
8	*S. epidermidis* and *S. hominis*	0	2	3	Cocci	2	+
9	*S. epidermidis* and *S. hominis*	1	2	3	Cocci	2	+
10	*S. epidermidis* and *S. capitis*	2	2	2	Cocci and *P. acnes*	1	+
11	*P. acnes*	2	2	3	*P. acnes*	1	+
12	*P. acnes*	1	1	1	*P. acnes*	1	+
13	*P. acnes*	NS[f]	NS	NS	*P. acnes*	3	+
14	*P. acnes*	0	2	3	Cocci and *P. acnes*	2	+
15	*P. acnes*	0	1	2	Cocci and *P. acnes*	2	+
16	*P. acnes*	2	2	3	Cocci and *P. acnes*	1	+
17	*P. acnes*	NS	NS	NS	Cocci and *P. acnes*	3	+
18	*P. acnes*	NS	NS	NS	Cocci and *P. acnes*	3	+
19	*P. acnes*	NS	NS	NS	Cocci and *P. acnes*	1	+
20	*P. acnes*	NS	NS	NS	Cocci and *P. acnes*	1	+
21	*P. acnes*	NS	NS	NS	Cocci and *P. acnes*	1	+
22	*P. acnes*	0	1	2	ND[g]	ND	+
23	*S. haemolyticus* and *P. acnes*	1	1	1	Cocci and *P. acnes*	1	+
24	*Micrococcus* sp. and *P. acnes*	1	2	3	Cocci and *P. acnes*	1	+
25	*S. epidermidis* and *P. acnes*	NS	NS	NS	Cocci	1	+

[a] Number of inflammatory cells per high-power field: 0, no cells; 1, 1 to 10 cells; 2, 10 to 20 cells; 3, 20 or more cells.
[b] PMN, polymorphonuclear leukocyte.
[c] LYM, lymphocyte.
[d] MAC, tissue macrophage.
[e] Number of bacteria per well: 0, no bacteria; 1, 1 to 10 bacteria; 2, 10 to 50 bacteria; 3, 50 or more bacteria.
[f] NS, not suitable for histological examination.
[g] ND, not done due to insufficient sample volume.

evidence of inflammatory cell infiltration in 29 samples. Two of the three samples in which tissue infiltration was not observed had IFM scores of 3, equivalent to 50 or more bacteria per well (Table 3). Neutrophilic infiltration was not observed in 12 of the 29 samples, although lymphocytes or macrophages were evident. A further eight samples were negative by 16S rRNA gene amplification but positive by immunolabelling, and one of these eight samples was also negative for inflammatory cell infiltration.

Seventeen samples were positive by 16S gene amplification but negative by IFM. Tissue pathology was available for only seven of these samples, and cell infiltration was observed in four samples. Tissue pathology results were available for 15 samples which were negative by both IFM and 16S rRNA gene detection. There was evidence of a giant cell-foreign body reaction in one of these, and the inflammatory cell score was zero for only one sample. Tissue pathology was not available for the remaining 12 culture-, IFM-, and 16S rRNA gene amplification-negative samples.

DISCUSSION

This study is the first to compare immunological and DNA detection methods with culture for the detection of bacterial infection of retrieved prosthetic hip joints. Bacteria were detected by immunolabelling and fluorescence microscopy with a *P. acnes*-specific MAb and polyclonal antiserum specific for *Staphylococcus* spp. only after the retrieved prostheses were subjected to mild ultrasonic treatment to dislodge the bacteria growing within a biofilm on the prosthetic joint surface. By this method bacteria were detected in all of the samples which were positive by culture and in 53% of the culture-negative samples. Bacteria, either coccoid or coryneform in morphology, were clearly visible by IFM. These were recorded as containing either *P. acnes* or *Staphylococcus* spp. due to the lack of cross-reactivity of the antibodies with other coccoid bacteria which are implicated in prosthetic implant infection, such as *P. magnus* (14) and other commensal and pathogenic coryneform bacteria. Therefore, the total number of samples positive by immunodetection was 71 of 113 (63%).

Previously, we have observed bacterial biofilms on retrieved prostheses by scanning electron microscopy (37); however, processing for scanning electron microscopy precludes examination of the biofilm by other methods. The use of CSLM in the present study showed that bacteria dislodged from implants by ultrasonication could be found in aggregates that varied in depth from 3 to 4.5 μm and that consisted of several layers of bacterial cells. It therefore seems that infecting bacteria growing within adherent biofilms are removed in large aggregates by the ultrasonication procedure. As the diluent used throughout was Ringer's salt solution, no enrichment was involved. Samples were also stored at −70°C; therefore, these aggregates could not have arisen as a result of multiplication of contaminating bacteria. It is worth noting that as these aggregates may contain over 300 bacteria, our estimates of the number of bacteria infecting an implant (37), calculated by total viable colony count per implant, are likely to be a gross un-

TABLE 3. Bacteria detected from culture-negative retrieved prosthetic hip implants by IFM and bacterial 16S rRNA gene detection and associated tissue inflammatory response[a]

Sample	Tissue infiltration by inflammatory cells (score)[b]			Immunofluorescence microscopy result		16S rRNA gene detection	Sample	Tissue infiltration by inflammatory cells (score)[b]:			Immunofluorescence microscopy result		16S rRNA gene detection
	PMN[c]	LYM[d]	MAC[e]	Morphology	Score[f]			PMN[c]	LYM[d]	MAC[e]	Morphology	Score[f]	
1	1	2	3	Cocci	3	+	38	3	1	1	*P. acnes*	1	−
2	1	2	3	Cocci	3	+	39	NS	NS	NS	*P. acnes*	1	ND[h]
3	0	2	0	Cocci	3	+	40	1	3	3	Cocci and *P. acnes*	3	+
4	0	0	0	Cocci	3	+	41	0	0	0	Cocci and *P. acnes*	3	+
5	3	3	2	Cocci	2	+	42	2	2	3	Cocci and *P. acnes*	2	+
6	1	3	3	Cocci	2	+	43	1	2	3	Cocci and *P. acnes*	2	+
7	0	2	3	Cocci	2	+	44	0	2	2	Cocci and *P. acnes*	2	+
8	0	1	1	Cocci	2	+	45	1	1	2	Cocci and *P. acnes*	1	+
9	1	2	3	Cocci	2	+	46	0	2	3	Cocci and *P. acnes*	1	+
10	0	2	3	Cocci	2	+	47	NS	NS	NS	Cocci and *P. acnes*	2	−
11	0	2	2	Cocci	1	+	48	1	2	3		0	+
12	0	1	3	Cocci	1	+	49	1	1	3		0	+
13	NS[g]	NS	NS	Cocci	2	+	50	1	1	2		0	+
14	NS	NS	NS	Cocci	2	+	51	0	1	1		0	+
15	NS	NS	NS	Cocci	1	+	52	0	0	0		0	+
16	1	2	3	Cocci	3	−	53	0	0	0		0	+
17	1	2	3	Cocci	3	−	54	0	0	0		0	+
18	1	2	1	Cocci	2	−	55	0	1	3		ND	+
19	NS	NS	NS	Cocci	3	−	56	0	1	2		ND	+
20	NS	NS	NS	Cocci	3	−	57	0	1	2		ND	+
21	2	2	3	*P. acnes*	2	+	58	0	0	3		ND	+
22	1	2	3	*P. acnes*	2	+	59	0	0	0		ND	+
23	2	2	3	*P. acnes*	1	+	60	2	3	3		0	−
24	2	2	3	*P. acnes*	1	+	61	1	3	3		0	−
25	3	2	2	*P. acnes*	1	+	62	1	2	3		0	−
26	1	2	3	*P. acnes*	1	+	63	1	2	3		0	−
27	1	2	3	*P. acnes*	1	+	64	1	2	2		0	−
28	0	1	2	*P. acnes*	1	+	65	0	2	3		0	−
29	0	1	2	*P. acnes*	1	+	66	0	2	3		0	−
30	1	1	1	*P. acnes*	1	+	67	1	1	2		0	−
31	0	1	1	*P. acnes*	1	+	68	1	1	1		0	−
32	0	0	1	*P. acnes*	1	+	69	0	1	1		0	−
33	0	0	0	*P. acnes*	1	+	70	0	1	1		0	−
34	NS	NS	NS	*P. acnes*	1	+	71	0	0	1		0	−
35	NS	NS	NS	*P. acnes*	1	+	72	0	0	1		0	−
36	NS	NS	NS	*P. acnes*	1	+	73	0	0	0		0	−
37	0	0	0	*P. acnes*	3	−							

[a] A further 21 samples not suitable for histological examination were negative by IFM, of which 10 were positive and 11 were negative by 16S rRNA gene detection.
[b] Number of inflammatory cells per high-power field: 0, no cells; 1, 1 to 10 cells; 2, 10 to 20 cells; 3, 20 or more cells.
[c] PMN, polymorphonuclear leukocyte.
[d] LYM, lymphocyte.
[e] MAC, tissue macrophage.
[f] Number of bacteria per well: 0, no bacteria; 1, 1 to 10 bacteria; 2, 10 to 50 bacteria; 3, 50 or more bacteria.
[g] NS, not suitable for histological examination.
[h] ND, not done due to insufficient sample volume.

derestimate. Each bacterial aggregate will give rise to only 1 CFU, and therefore, the total number of bacteria colonizing the implant will be considerably more than the number of CFU counted. Examination for potential skin-flake contamination, generated by scraping and rubbing the skin surfaces of laboratory personnel, showed that bacteria present as a result of this type of contamination should have been easily distinguishable by IFM from bacterial aggregates dislodged from the prosthesis biofilm. Skin cells were not observed in the sonicates. Examination of skin scrapings revealed few bacteria and no large aggregates. The estimated density of *P. acnes* ranges from 10^2 to 10^5 per cm^2 of skin (26), and propionibacteria account for approximately half of the total skin microbiota (35). It would therefore take gross skin contamination for this to be visible by immunofluorescence microscopy. Contamination of the prostheses by the skin of the patient, surgical operating staff, or laboratory personnel should have easily been detectable. Furthermore, if prostheses were placed in diluent which was then aspirated, without sonication, bacterial aggregates were not detected in the diluent. If prostheses were placed in fresh diluent and then sonicated, bacteria could be observed in some samples. Sonication was therefore necessary to remove the adherent bacteria, which again makes it highly unlikely that the bacteria detected arose from skin contaminants. The contamination of clinical samples by the normal skin microbiota is a potential problem in clinical diagnostic procedures involving an enrichment step in which small numbers of contaminants have the opportunity to multiply along with infecting microbes. We have clearly shown that when no enrichment step is used and samples are processed in Ringer's solution rather than broth diluent, it is possible to distinguish between infecting and contaminating microbes. This is proba-

bly largely due to quantitative differences; if there are any contaminating bacteria they are few in number compared to the number of infecting microbes.

Bacterial 16S rRNA was detected in all culture-positive samples by use of two broad-range oligonucleotide primers that had been designed in order to amplify by PCR rRNA genes from a wide spectrum of bacteria implicated in prosthetic hip infection. Bacterial 16S rRNA genes (termed PCR positive) were detected in all of the culture-positive samples and in a further 65% of the culture-negative samples. In total, 85 of 118 (72%) samples were positive by PCR. Culture-negative, PCR-positive samples are unlikely to represent samples with false-positive results as suitable stringent control procedures were carried out to ensure that such samples were not positive due to the presence of contaminating bacterial DNA (4, 27). The lower limit of detection was also estimated to be approximately 10^4 CFU/ml. Samples were therefore unlikely to be positive as a result of exogenous contamination. Various methods could have been used to increase the efficiency of the PCR, including the use of nested PCR; however, as these should also have increased the chance of detecting contaminants, they were not used. In a comparison of culture and 16S rRNA gene detection methods for detection of total knee arthroplasty infection, a similar level of infection was reported. Evidence of bacterial infection on the basis of PCR amplification of synovial fluid aspirates was reported for 32 of 50 specimens (64%) (25).

Although the data for the tissue pathology are incomplete, both the rRNA gene detection and IFM results related well to the presence of inflammatory cells in implant-associated tissue; in only three instances was the inflammatory cell score zero. In two of these the IFM score was high, which suggests that the negative tissue pathology result may have been due to inadequate tissue sampling (1).

In some instances, although the tissue pathology score was high, bacteria were only evident in low numbers by IFM. It may be that some of the bacteria are strongly adherent to the device and sonication is insufficient to remove them, an observation we have made with in vitro models of biofilms (38). Also, in some cases the bacteria may predominantly colonize bone rather than the prosthesis. Alternatively, any infecting bacteria may not have reacted with our sera. We are addressing these issues. The lack of observation of neutrophilic infiltration in eight of the culture-positive samples, in which lymphocytes or macrophages were present, suggests that neutrophil infiltration should perhaps not be used as the sole indicator of infection.

It is likely that prosthetic joint infection may be quite variable in nature, depending on the type of infecting bacteria, whether the infection is polymicrobial, and the length of time that the implant has been infected. When bacteria are prevalent in the surrounding tissues, the infection may be typified by high levels of neutrophil infiltration in tissue; however, when the majority of bacteria are growing on the device surface within a biofilm, the infection may be typified by lymphocyte and macrophage infiltration. Within the tissues, cells of the immune system will come into contact with living whole bacterial cells, whereas it is likely that the major immunostimulants of biofilm bacteria will be secreted or released products. An immunoglobulin response to polysaccharides, thought to be mainly teichoic acids, released by *S. epidermidis* is detectable in the serum of patients with prosthetic joint infection (21). Teichoic acids are well recognized as belonging to the group of immunomodulatory molecules which may be present in or released from the bacterial cell envelope (31). Whether the bacteria involved in these infections, other than *S. aureus*, are able to produce other immunomodulatory molecules, such as superantigen toxins, is unknown. The immunostimulatory properties of *P. acnes* are well documented and comparable to those of bacteria such as *Mycobacterium tuberculosis* and *Bordetella pertussis* (11). Therefore, low-grade chronic infection with *P. acnes* could be sufficient to stimulate the observed inflammatory cell infiltration. A lack of a neutrophilic inflammatory response is characteristic of diseases such as typhoid and tuberculosis, which are caused by bacteria which grow intracellularly (33). Interestingly, there are reports in the literature that *P. acnes* can also grow intracellularly (11). It should also be noted that the presence of giant cells without evidence of neutrophils, although indicative of reaction to the device materials (28), may not necessarily rule out bacterial infection of the device.

The detection of bacteria in sonicates from patients with culture-negative implants indicates that these implants were colonized by bacteria which were not isolated by the microbiological techniques used. This may be because the implants were infected with viable but nonculturable bacteria. Interestingly, in some instances only one type of bacterium was detected by culture, but both coccoid and coryneform bacteria were observed by IFM. The possibility that the bacteria were nonculturable as a result of the cefamandole administered intravenously at the time of operation must be addressed, although it is generally considered that bacteria growing within an adherent biofilm on the implant surface are protected from antibiotic therapy (36). We examined the antibiotic sensitivities of the bacteria that we isolated from prostheses. The majority were approximately 1,000 times more resistant (minimum bactericidal concentration, >1,024 μg/ml) to cefamandole when they were growing within in vitro model biofilms than when they were growing in broth culture (39, 40). This indicates that it is unlikely that the cefamandole rendered the bacteria unculturable. Naylor et al. (29) and Costerton et al. (7) have previously proposed that alterations of bacterial metabolism may be responsible for the enhanced antibiotic resistance of bacteria growing within a biofilm.

The detection of bacterial DNA in culture-negative clinical samples by PCR amplification despite prolonged antibiotic therapy resulted in several researchers concluding that antibiotic administration did not eradicate the infecting bacteria but rendered them nonculturable. For example, Canvin et al. (5) described a case of septic arthritis in a patient with rheumatoid arthritis and prosthetic knee joints. Cultures of synovial fluid from the patient's knees were initially positive for *S. aureus* but rapidly became sterile after 1 week of antibiotic therapy. However, synovial fluid samples taken until 10 weeks of therapy showed the persistence of *S. aureus* by the presence of specific staphylococcal DNA by PCR, leading the investigators to conclude that either nonviable debris persisted after infection or that organisms were still viable but rendered nonculturable by antibiotic administration. Similarly, Ni et al. (30) used PCR to detect meningococcal DNA in 54 cerebrospinal fluid samples for which antibiotic administration had prevented isolation by conventional culture techniques. In the present study, the observation of whole bacterial cells by IFM indicates that our samples do not only contain bacterial DNA debris. Another possible explanation for the lack of viability is that the bacteria from the implants are sensitive to mild ultrasonication. Although pure cultures of laboratory-grown bacteria are not killed by this level of ultrasound (37), bacteria which have been growing in vivo and subsequently stressed by removal from the patient and transportation to the laboratory for processing may have greater sensitivity.

It is also possible that viable but nonculturable bacteria are so highly adapted to the environment of the in vivo biofilm that the conditions required for their continued growth, and there-

fore successful isolation, are not met by the growth media and isolation procedures used. In effect, growth within the in vivo biofilm renders the bacteria more fastidious with respect to growth requirements. One possible explanation for the difficulty in the isolation of these bacteria could be the dilution of bacterial signaling molecules, of which a critical concentration may be necessary to trigger growth of the bacteria. Quorum sensing, involving, for example, *N*-acyl homoserine lactones in gram-negative bacteria (8) and peptide pheromone in gram-positive bacteria (22), is well characterized. Whether prolonged growth in this environment also results in irreversible genotypic changes remains an open question. Bacterial pathogens subjected to the highly variable challenge of the human immune system are well known for genetically based reversible variation (31); however, bacteria growing for long periods in what may be a relatively static environment, largely protected from the immune system, may, in the long term, become less adaptable.

Advantages and disadvantages are associated with the use of both 16S rRNA gene amplification and immunolabelling to detect infection. Benefits of both methods include rapid detection of infection when culture techniques have proved ineffective and the ability to detect bacteria in clinical samples in the presence of antimicrobial drugs. The major advantage of rRNA gene detection is that the use of universal broad-range primers allows recognition of any bacteria that may be present and polymicrobial infections can be detected as it is not necessary to predict which bacteria may be present. In contrast, for immunological detection of infection to be effective, the MAbs and polyclonal antiserum must be prepared against the bacteria thought to cause the infection, and other bacteria will therefore not be detected. This could explain why more culture-negative samples were positive for infection by rRNA gene detection than by immunolabelling in the present study. As well as being a disadvantage, this requirement can also be an advantage as the identity of the infecting bacteria can be ascertained immunologically. Also, direct visualization of the bacterial morphology makes it very unlikely that a false-positive diagnosis will be made or, indeed, that contaminating bacteria will be mistaken for infecting bacteria. In contrast, bacterial 16S rRNA gene amplification alone does not identify the infecting bacteria. For definitive identification of infecting bacteria, the amplification products could be directly sequenced or, in the case of mixed infections, first cloned into *E. coli* plasmid vectors and then sequenced.

In conclusion, this study implicates unrecognized infection as a potential major cause of prosthetic hip failure. IFM allows a rapid quantitative and qualitative assessment of infected prostheses and distinguishes the bacteria from the infected prostheses from bacteria that may result from skin contamination. The IFM results indicate that 63% of retrieved hip prostheses may be colonized with bacteria. 16S rRNA genes were detected from 72% of retrieved prostheses. We are investigating the nature of the bacteria detectable by PCR amplification but not by IFM. The follow-up of these patients with respect to the successes of the second prostheses is a critical part of our study; however, the treatment of patients with primary prostheses must be addressed in the short term, given the long-term nature of this type of infection. Unfortunately, neither of the nonculture detection methods allows the determination of antimicrobial susceptibility. This limitation can be partly overcome, however, by using current knowledge of prosthetic hip infection as a guide to the expected pathogens and their antimicrobial susceptibilities, in particular when they are growing within biofilms. The improved detection of infection could then be coupled with appropriate antibiotic therapy for a longer postoperative period, and this should improve the clinical outcomes for patients undergoing revision hip surgery.

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In: Biofilms. RJ Doyle (Ed) Methods in Enzymology 310, 566-576.

determined by the portion of the urinary tract in which the test biomaterial is destined to reside. For example, there will be more urine flow in the ureters than in the more stagnant portions of the bladder. Therefore, a biomaterial being tested for use as a ureteral stent should be tested in more dynamic conditions to mimic the flow of urine over the biomaterial surface *in vivo*. Models such as those described within, especially where urine flow is involved, will prove useful for testing novel biodegradable biomaterials for use as urinary prostheses. In practice, urine flow may encourage surface layers of the degradable polymer to be shed, thereby assisting the removal of encrusting deposits and adherent microorganisms. Such biomimetic biomaterials may have a role to play in the development of improved urinary tract devices.

[42] Detection of Prosthetic Joint Biofilm Infection Using Immunological and Molecular Techniques

By MICHAEL M. TUNNEY, SHEILA PATRICK, MARTIN D. CURRAN, GORDON RAMAGE, NEIL ANDERSON, RICHARD I. DAVIS, SEAN P. GORMAN, and JAMES R. NIXON

Introduction

Total hip replacement is one of the most successful and cost-effective surgical operations ever devised, with over 50,000 hip replacements performed annually in the United Kingdom and 200,000 in the U.S. The majority of patients who undergo hip replacement experience dramatic relief of preoperative pain and restoration of satisfactory hip function.[1] A proportion (approximately 20% in Europe)[2] fail, and prosthesis removal and replacement (revision hip arthroplasty) is usually required with further trauma for the patient and increased cost to the Health Service.[3] Studies have reported that between 2[4] and 15%[5] of all revision operations result from infection of the implant. Unfortunately, the rate of infection is higher after revision hip arthroplasty than after primary procedures, with as many as 40% of revised hip joints failing due to infection.[6] It has been suggested

[1] R. H. Fitzgerald, *Orthop. Clin. N. Am.* **23,** 259 (1992).
[2] P. Christel and P. Djian, *Curr. Opp. Rheum.* **6,** 161 (1994).
[3] C. R. Dreghorn and D. L. Hamblen, *Br. Med. J.* **298,** 648 (1989).
[4] R. L. Barrack and W. H. Harris, *J. Bone Joint Surg.* **75,** 66 (1993).
[5] P. F. Lachiewicz, G. D. Rogers, and H. C. Thomason, *J. Bone Joint Surg.* **78,** 749 (1996).
[6] J. A. Dupont, *Clin. Orthop. Relat. R* **211,** 122 (1986).

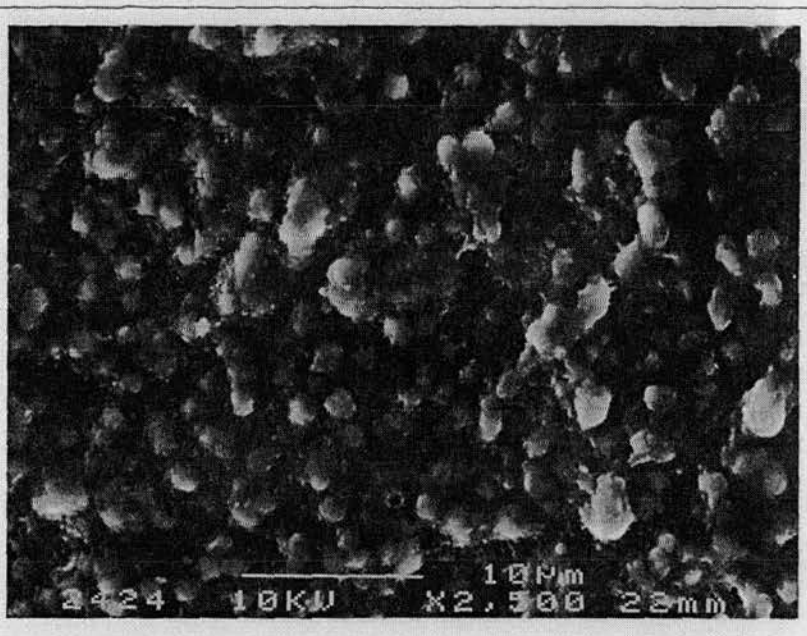

FIG. 1. Scanning electron micrograph showing bacterial biofilm on a retrieved orthopedic implant.

that this higher rate of infection postrevision may be due to unrecognized infection at the initial revision operation.[6] This may be because bacteria colonizing the surface of implanted biomaterials grow predominantly in adherent biofilms[7] (Fig. 1) and may not be detected by aspiration and routine culture techniques, which fail to examine the retrieved prostheses.[8] Additionally, given the well-proven incidence of anerobic bacteria in joint infection,[9] it is likely that this is also due to the fastidious culture requirements of anaerobic bacteria.

This article describes how the detection of bacteria by culture from revision hip prostheses can be improved by employing strict anaerobic techniques and by using mild ultrasonication to dislodge bacteria adhering to the surface of the retrieved implants. Nonculture techniques that can be used to further improve the detection of infection, including examination of the inflammatory response in implant-associated tissue and the detection of bacteria using immunological and polymerase chain reaction (PCR)-based molecular approaches, are also described.

[7] M. M. Tunney, S. P. Gorman, and S. Patrick, *Rev. Med. Microbiol.* **7,** 195 (1996).
[8] A. G. Gristina and J. W. Costerton, *J. Bone Joint Surg.* **67,** 264 (1985).
[9] I. Brook and E. H. Frazier, *Am. J. Med.* **94,** 21 (1993).

Improved Culture Technique for Detection of Infection

Current clinical laboratory practice for the detection of prosthetic hip infection involves the culture of bacteria from joint fluid aspirated peroperatively and from tissue samples removed at the time of surgery. A previous study has shown that mild ultrasonication of retrieved tissue, bone, and cement can increase the number of bacterial isolates cultured compared with the numbers cultured from joint fluid or swabs of excised tissue and prosthetic surfaces.[8]

Sample Collection

1. When removed from the patient, the femoral and acetabular components of the prosthetic hip are placed aseptically in separate sterile bags.
2. Tissue in contact with the implants is also removed and placed in sterile bottles.
3. The femoral and acetabular components of the prostheses and the tissue samples are placed immediately in an anaerobic jar (e.g.,Oxoid Anaerobic Jar HP11, Unipath Ltd., Basingstoke, UK) containing a catalyst (e.g., Oxoid Low Temperature Catalyst BR42, Unipath Ltd.) and a Gaspak (e.g., Oxoid Gas Generating Kit BR038B, Unipath Ltd.) is added immediately.
4. The jar is transported to the laboratory and placed in an anaerobic cabinet (e.g., Don Whitley Mk III, Don Whitley Scientific Ltd., Shipley, UK) containing an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 (v/v/v), where it is opened.

Sample Processing

The two parts of the prostheses are processed independently.

Prostheses

1. Ringer's solution (25%, v/v) is prereduced by the addition of L-cysteine (1%, v/v) and by the subsequent removal of oxygen by either boiling at a temperature of 100° for 1 hr in a water bath or microwaving at full power (650 W) for 5 min. On cooling, the Ringer's solution is stored in the anaerobic cabinet prior to use.
2. Separate 100-ml volumes of prereduced Ringer's solution are added to the sterile bags containing the prostheses.
3. The prostheses are removed from the cabinet inside the sealed bags and any material attached to the prosthesis surface is dislodged into the Ringer's solution using mild ultrasonication (5 min in a 150-W

ultrasonic bath operating at a nominal frequency of 50 Hz). Ringer's solution is removed from the sealed bags and is placed in sterile bottles that are immediately transferred to the anaerobic cabinet. It is essential that this part of the procedure be performed quickly and efficiently to minimize exposure to air of any anaerobic bacteria present.

4. Total viable bacterial counts are performed by spreading 0.5-ml volumes of sonicate onto five blood agar [BA; 40 g/liter, Oxoid blood agar base No. 2, Unipath Ltd.; 5% (w/v) Oxoid horse blood, Unipath Ltd.] and five anaerobic blood agar (ABA; 47 g/liter, GIBCO anaerobic blood agar base, GIBCO Ltd., Paisley, UK) plates, which are stored in the anaerobic cabinet prior to use.
5. To enhance detection of bacteria that may be present in low numbers, two 5-ml volumes of each sonicate are concentrated using separate Millipore Sterifill units [Millipore (UK) Ltd., Watford, UK] and separate 0.22-μm filters (Supor-200 membrane filters, Gelman Sciences, Ann Arbor, MI), which are then placed on both a BA and an ABA plate.
6. The remaining 85 ml of each sonicate is centrifuged for 20 min at 10,000*g* and the pellet produced is resuspended in 3 ml Ringer's solution, which is then dispensed into 1-ml volumes that are stored at −70°.

Tissue

1. A known weight of tissue is homogenized for 3 min in 5 ml prereduced Ringer's solution using a mechanical homogenizer, and total viable bacterial counts are performed by spreading 0.5-ml volumes of homogenized tissue onto three BA and three ABA plates.
2. To detect bacteria present in low numbers, the remaining volume of homogenized tissue is added in equal amounts to tryptone soya broth (TSB; 30 g/liter, Oxoid tryptone soya broth, Unipath Ltd.) and cooked meat broth (CMB; 100 g/liter, Oxoid CM 81, Unipath Ltd.) for enrichment.

Bacterial Culture and Identification

BA and ABA plates are incubated at 37° aerobically and anaerobically, respectively, and examined after 1, 2, 4, and 7 days. The TSB and CMB are incubated at 37° aerobically and anaerobically, respectively, and subcultured onto both BA and ABA after 7 and 14 days. These plates are then incubated either aerobically or anaerobically at 37° for another 7 days.

All bacteria cultured are counted to allow direct quantitation of the number of infecting bacteria. Pure cultures of any bacteria isolated are

gram stained and identified using commercially available biochemical test galleries (e.g., API20A, APIStaph, Biomerieux, Paris, France).

Nonculture Techniques for Detection of Infection

Histological Examination of Tissue

Assessment of histopathological reactions in tissue samples can be quantified easily and it has been shown that the presence of acute or chronic inflammation in tissue samples correlates well with culture results and can, therefore, be used to aid diagnosis of infection.[10–12]

1. Selected tissue samples are fixed in formal saline histological fixative (Gurr, BDH, Davidson and Hardy Ltd., Belfast, UK). Representative samples are then processed and embedded in paraffin wax, and 5-μm sections are cut and stained using a hematoxylin and eosin stain.
2. All slides are subsequently assessed microscopically without prior knowledge of culture results using the following technique. The hematoxylin and eosin stained sections are scanned at low power (×100 objective) and the areas of the slide containing the heaviest inflammatory infiltrate are selected for further examination at a higher magnification (×400 objective). If the sample is considered satisfactory, the number of inflammatory cells per high power field is assessed over five randomly selected fields.
3. The following parameters are assessed in each tissue sample:

   a. Acute inflammatory response (infiltration with polymorphonuclear leukocytes).

   b. Chronic inflammatory response (infiltration with lymphocytes and tissue macrophages).

For each type of inflammatory cell, the slide is graded according to the following scheme:

0	absent
1	1–10 cells/high power field
2	10–20 cells/high power field
3	20 or more cells/high power field

[10] T. K. Fehring and J. A. McAlister, *Clin. Orthop. Relat. R* **304,** 229 (1994).
[11] J. M. Mirra, H. C. Amstutz, M. Matos, and R. Gold, *Clin. Orthop. Relat. R* **117,** 221 (1976).
[12] W. J. Kraemer, R. Saplys, J. P. Waddell, and J. Morton, *J. Arthroplasty* **8,** 611 (1993).

Molecular Detection of Infection

The detection of bacterial 16S ribosomal RNA (rRNA) genes as an indicator of the presence of bacteria is a recognized technique that has been used for the detection of both environmental and medically important bacteria.[13,14] Because bacterial rRNA gene sequences contain regions that are conserved within the prokaryotes and regions that are unique to different bacterial genera and species, they provide an ideal tool for the specific detection and identification of bacteria. The availability of a growing database of rRNA sequences and the ability to amplify rRNA-encoding genes (rDNA) from the limited amount of material that is available for analysis via PCR further enhance the suitability of rRNA sequences for detection and subsequent identification of bacteria.[15]

DNA Extraction

All steps are carried out in 1.5-ml Eppendorf tubes.

1. Centrifuge a 1-ml amount of prosthesis sonicate at 10,000*g* for 15 min at room temperature. Discard the supernatant.
2. Lysozyme (1–2 mg/ml) in 10 m*M* Tris (pH 8.0) and 50 m*M* glucose can also be used to help in the extraction but attention to contamination with exogenous bacterial DNA must be taken into account.
3. Suspend pelleted cells by vortex mixing in 200 μl cell lysis buffer [containing 10 m*M* Tris (pH 8.0), 5 m*M* EDTA, 0.5% sodium dodecyl sulfate (SDS), and proteinase K (100 μg/ml)] and incubate at 55° for 3 hr.
4. Adjust the temperature to 37° and leave reaction mixture overnight.
5. Increase the temperature of reaction mixture to 55° for 1 hr.
6. Add an equal volume of saturated phenol/chloroform, vortex thoroughly, and centrifuge at 10,000*g* for 15 min at room temperature.[16] Repeat this step if necessary.
7. Remove the aqueous supernatant to a new tube, being careful to leave the interface behind.
8. Add ethanol (100%, 2.5 volumes), 3 *M* sodium acetate, pH 5.2 (0.1 volumes), and 2 μl of See DNA® (Pharmacia Biotech, Piscataway, NJ). Vortex briefly and centrifuge at 10,000*g* for 15 min at room temperature.

[13] K. Griesen, M. Loeffelholz, A. Purohit, and D. Leong, *J. Clin. Microbiol.* **32,** 335 (1994).
[14] M. J. Wilson, A. J. Weightman, and W. G. Wade, *Rev. Med. Microbiol.* **8,** 91 (1997).
[15] T. M. Schmidt and D. A. Relman, *Methods Enzymol.* **235,** 205 (1994).
[16] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

9. Pour off supernatant and wash the pelleted DNA with 1 ml of 70% (v/v) ethanol. Vortex briefly and centrifuge at 10,000*g* for 15 min at room temperature.
10. Remove the supernatant and dry the pellet in a vacuum dryer (DNA Plus, Heto Laboratory Equipment).
11. Dissolve extracted DNA in 50 μl of TE [10 m*M* Tris (pH 8.0), 1 m*M* EDTA] buffer and store at −20°.

Oligonucleotide Primer Design

Using DNAstar computer software (Megalign), alignments of eubacterial 16S rRNA gene sequences can be constructed. From these it is possible to improve the range of previously published broad-range PCR primer pairs[17] by adding in a number of degeneracies in the primers. An example of primer sequences that can be used are as follows:

D1: 5′-GAG GAA GGT RGG GAY GAC GT
D2: 5′-AGG CCC GGG AAC GYA TTY ACC G
R = AG, Y = CT.

Both primers flank a hypervariable region that aids in 16S rRNA microbial identification.

Polymerase Chain Reaction Amplification

The PCR mixture is made up to 50 μl in sterile water and contains 5 μl of 10× PCR buffer (Perkin-Elmer, Norwalk, CT), 5 μl $MgCl_2$ (25 m*M*), 200 μM of each deoxynucleotide triphosphate (Pharmacia Biotech, Piscataway, NJ), 20 p*M* of each primer, 3 units of Ampli*Taq* polymerase (Perkin-Elmer Corporation, UK), and 2 μl of lysate containing target DNA.

The typical PCR run profile is a 5-min denaturation of 96°, followed by 30 cycles of 1 min at 96°, 2 min at the annealing temperature of 55°, and 1 min at 72°. The final cycle is ended with a 5-min extension at 72°, and the reaction is then held at 15° until the tubes are removed from the thermocycler (Perkin-Elmer Gene-Amp PCR System 9600; Perkin-Elmer Corporation, UK). After amplification, 6 μl of the amplified product is run on a 1.5% (w/v) agarose gel in 1× Tris–borate–EDTA (TBE). DNA bands are detected by ethidium bromide staining and visualized by UV light photography.

The sensitivity of the extraction procedures should be investigated by extracting DNA from 10-fold serial dilutions of pure cultures and subse-

[17] M. N. Widjojoatmodjo, A. C. Fluit, and J. Verhoef, *J. Clin. Microbiol.* **33**, 2601 (1995).

quently determining the limits of detection following PCR amplification and agarose gel electrophoresis.

Single-Stranded Conformational Polymorphism Electrophoresis

Single-stranded conformational polymorphism (SSCP) is a simple method that can be used to detect nucleotide sequence changes in PCR products.[18] It is ideally suited for fingerprinting the hypervariable sequences of the 16S rRNA of eubacteria, providing a rapid preliminary identification for any PCR-positive samples. It also allows the detection of polymicrobial infections, as more than one SSCP profile will be apparent. The following is a brief outline of the procedure.

1. After thermal cycling, 4 μl of the PCR mixture is added to 11 μl of sequencing sample buffer [5 m*M* EDTA (pH 8.0), 0.25% (w/v) bromphenol blue, in deionized formamide] and heated for 5 min at 96°.
2. The denatured DNA is then placed directly on ice for 10 min before being applied to the gel in 10-μl volumes. Bacterial standards are also applied to the gel for direct comparison. The optimal gel composition is 0.5× mutation detection enhancement gel (Flowgen, Shenstone, Staffordshire, UK), 0.6× TBE, 0.04% (w/v) ammonium persulfate solution, and 0.004% (v/v) *N,N,N′,N′*-tetramethylethylenediamine.
3. Electrophoresis is performed at 23° on a Sequi-Gen, vertical gel electrophoresis apparatus (Bio-Rad, Richmond, CA) for 5 hr at 20 W constant power.
4. After electrophoresis, SSCP patterns are detected by silver staining according to the method of Bassam *et al.*[19] Briefly, the procedure is as follows.

a. Gels are fixed in 10% (v/v) glacial acetic acid for 30 min at room temperature and washed with deionized water four times for 2 min each.

b. Gels are then color impregnated for 30 min at room temperature with 0.1% (w/v) silver nitrate and 0.056% (v/v) formaldehyde.

c. Gels are then washed for 30 sec with deionized water prior to color development. Color development is for 2 to 10 min with a mixture of 30 g/liter sodium carbonate, 0.056% (v/v) formaldehyde, and 4 mg/liter sodium thiosulfate. The color reaction is stopped by the addition of 10% (v/v) glacial acetic acid.

[18] J. R. Kerr and M. D. Curran, *Clin. Mol. Pathol.* **49,** 315 (1996).
[19] B. J. Bassam, G. Caetano-Anolles, and P. M. Gresshoff, *Anal. Biochem.* **196,** 80 (1991).

DNA Sequencing

For definitive identification of bacteria, the PCR amplification products are sequenced directly by the dideoxynucleotide chain termination procedure using the PRISM Ready Reaction Terminator Cycle Sequencing Kit and read using a Model 373A automated sequencer (Applied Biosystems Inc.).

Immunological Detection of Infection

Direct immunological detection of bacteria in clinical samples can be achieved by the use of monoclonal antibodies (MAbs) and polyclonal antiserum prepared against the bacteria implicated in the clinical infection.[20]

Detailed methods describing the production of polyclonal antiserum and MAbs can be found in the laboratory manual of Harlow and Lane[21] and the textbook of Goding.[22] A brief description of both techniques follows.

Production of Polyclonal Antiserum

A New Zealand White rabbit is immunized with whole cells of bacteria. The rabbit is inoculated subcutaneously at four sites on the back with 0.1 ml of a bacterial suspension of 1×10^8 cfu (colony-forming units)/ml in 0.01 *M* phosphate-buffered saline (PBS). A further two inoculations of bacteria in PBS are made at approximately monthly intervals and the rabbit is test bled 2 weeks after the final booster dose. The blood is allowed to clot at 37° for 1 hr and contract at 4° overnight after which the serum is tested by immunofluorescence microscopy.

Production of MAbs

A BALB/c mouse is immunized by whole bacterial cells. The mouse is inoculated intraperitoneally with 0.2 ml of a bacterial suspension of 1×10^8 cfu/ml in 0.01 *M* PBS. A further inoculation of 0.2 ml is given 4 days before the mouse is killed. Spleen cells from the mouse are fused with P3X 63 Ag8-653 (NS-0/1) mouse myeloma cells by treatment with 50% polyethylene glycol 16000 (Sigma, Dorset, UK) in RPMI 1640 (Flow Labo-

[20] S. Patrick, L. D. Stewart, N. Damani, K. G. Wilson, D. A. Lutton, M. J. Larkin, I. Poxton, and R. Brown, *J. Med. Microbiol.* **43,** 99 (1995).

[21] E. Harlow and D. Lane, "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.

[22] J. W. Goding, "Monoclonal Antibodies: Principle and Practice." Academic Press, London, 1986.

ratories, Paisley, UK) using a modification of the method described by Galfre and Milstein.[23] Hybrid cell lines are selected with hypoxanthine–aminopterin–thymidine in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 20% myoclone fetal calf serum (GIBCO). Culture supernatants are removed and screened by immunoblotting and immunofluorescence for IgG specific for the bacteria used for inoculation.

Immunofluorescence Microscopy

For further information on this technique, readers are directed to the detailed description of the technique provided by Patrick and Larkin.[24] The method in brief is as follows.

1. One-milliliter samples of prosthesis sonicate are centrifuged (10,000*g*, 15 min, room temperature) and the resulting pellets suspended in 100 μl PBS.
2. Samples (10 μl) are then applied in duplicate to multiwell slides. The slides are air-dried and fixed in methanol (100%, 10 min) at −20°, after which they may be stored for up to 6 months at −20°.
3. Samples are then examined by dual labeling using the following procedure:

   a. Incubate the slides in a humidified box with undiluted MAb supernate for 45 min.

   b. Wash slides briefly with a wash bottle containing PBS and then wash for 30 min in a bath containing PBS.

   c. Incubate slides with polyclonal rabbit antiserum diluted in PBS for 45 min and repeat washing step.

   d. Incubate slides for 45 min simultaneously with goat antimouse rhodamine conjugate (Sigma, Poole, UK) and sheep antirabbit fluorescein conjugate (Sigma), both diluted in PBS as recommended by the manufacturer.

   e. After a final wash, mount slides with glycerol-PBS containing an antiphotobleaching agent (e.g., Citifluor, Agar Scientific Ltd., Essex, UK) and examine using a fluorescence microscope (e.g., Leitz fluorescence microscope) with filters suitable for examining fluorescein and rhodamine separately and in combination.

[23] G. Galfre and C. Milstein, *Methods Enzymol.* **73,** 1 (1981).

[24] S. Patrick and M. J. Larkin, *in* "Microbial Biofilms: Formation and Control" (S. P. Denyer and S. P. Gorman, eds.), p. 109. Blackwell Scientific Publications, Oxford, 1993.

4. For detection of bacteria on each well by immunofluorescence microscopy, a score is given of between 0 and 3 using the following criteria:

0	absent
1	1–10 bacteria/well
2	10–50 bacteria/well
3	50 or more bacteria/well

Concluding Remarks

The use of strict anaerobic techniques and mild ultrasonication described herein has resulted in bacteria being cultured from 26 of 120 (22%) retrieved implants examined.[25] Review of the notes from 18 of these 26 individuals revealed that infection prior to revision was only suspected in 6 cases and that in only 2 of these cases were bacteria cultured from preoperative aspirates or tissue removed at the time of surgery using conventional microbiological techniques.

We have also used the nonculture techniques described in this article to detect infection of retrieved prosthetic hip joint.[26] Results obtained using these techniques confirmed those obtained using bacterial detection by culture following mild ultrasonication of the implants, with all culture-positive samples immunofluorescence microscopy-positive and positive for the presence of bacterial 16S rRNA genes and associated tissue samples positive for the presence of inflammatory cells indicative of infection.[25] Their use also revealed the presence of bacteria in a large number of culture-negative samples, with 68% having detectable bacterial rRNA gene sequences, 53% having a positive immunofluorescence result, and 87% of associated tissue samples positive for the presence of inflammatory cells indicative of infection.[25] The use of these nonculture techniques to improve the detection of prosthetic joint biofilm infection coupled with appropriate postoperative antibiotic therapy should improve the clinical outcome for patients undergoing revision hip surgery.

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Detection of Intrastrain Antigenic Variation of *Bacteroides fragilis* Surface Polysaccharides by Monoclonal Antibody Labelling

SHEILA PATRICK,* DEIRDRE GILPIN, AND LEANNE STEVENSON

Department of Microbiology and Immunobiology, School of Medicine, Queen's University of Belfast, Belfast BT12 6BN, United Kingdom

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***Bacteroides fragilis* is a constituent of the normal resident microbiota of the human intestine and is the gram-negative obligately anaerobic bacterium most frequently isolated from clinical infection. Surface polysaccharides are implicated as potential virulence determinants. We present evidence of within strain immunochemical variation of surface polysaccharides in populations that are noncapsulate by light microscopy as determined by monoclonal antibody labelling. Expression of individual epitopes can be enriched from a population of an individual strain by use of immunomagnetic beads. Also, individual colonies in which either >94% or <7% of the bacteria carry an individual epitope retain this level of expression when subcultured into broth. In broth cultures where >94% of the bacteria carry a given epitope, there is no enrichment for other epitopes recognized by different polysaccharide-specific monoclonal antibodies. This intrastrain variation has important implications for the development of potential vaccines or immunodiagnostic tests.**

Bacteroides fragilis is the gram-negative strictly anaerobic bacterium most frequently isolated from clinical infection. The major source of these infections is the normal resident colonic microbiota, where *Bacteroides* spp. outnumber facultatively anaerobic bacteria such as *Escherichia coli* by a factor of between 10^2 and 10^3 (4). In the fecal microbiota, the predominant *Bacteroides* spp. is *B. vulgatus*, with *B. fragilis* a relatively minor component. Namavar and colleagues (6) report a relatively higher proportion of *B. fragilis* in the adherent mucosal microbiota; however, this was not confirmed by Poxton and colleagues (19). It therefore appears that the frequency with which *B. fragilis* is isolated from infection compared to other *Bacteroides* spp. of the resident microbiota cannot be explained simply by weight of numbers.

The potential virulence determinants of *B. fragilis* have been the subject of many investigations (9). It is clear that a number of factors may contribute to the virulence of *B. fragilis*, including surface structures, release of extracellular enzymes, iron-scavenging mechanisms, and enterotoxin production; however, extracellular polysaccharides have been considered to play a key role in *B. fragilis* virulence. Encapsulating structures have been implicated in resistance to complement-mediated killing, phagocytic uptake, and killing (21) and abscess formation in an animal model (27). Many studies have failed to take into account not only within-strain variation in capsule production but also between- and within-strain antigenic variation of different types of capsules. By electron microscopy, it is possible to identify within an individual strain of *B. fragilis* bacteria with either large or small capsules which are fibrous in appearance but are antigenically different, as well as bacteria with an encapsulating electron-dense layer (EDL) adjacent to the outer membrane (15, 16). The EDL bacteria are noncapsulate by light microscopy, whereas the small and large capsules are clearly visible with negative staining. Expression of the different capsular types is inheritable as populations can be enriched by subculture from different interfaces of Percoll step density gradients. Microscopical observation of the populations enriched for the three capsular types with monoclonal antibodies (MAbs) specific for surface polysaccharides shows that noncapsulate bacteria are antigenically different from bacteria with the small capsules but have shared epitopes with large-capsule bacteria. In addition, immunofluorescent and immunogold labelling for fluorescence and electron microscopy, respectively, reveals antigenic variation in populations which appear to be structurally homogeneous (5, 13, 22, 23). This phenomenon has been observed in recent clinical isolates from a variety of anatomical sites, in isolates from different geographical locations, and in culture collection type cultures (17). By polyacrylamide gel electrophoresis (PAGE) and immunoblotting with MAbs specific for surface polysaccharides, distinctive patterns are observed within the noncapsulate population of an individual strain. These results indicate that an individual *B. fragilis* strain may produce a number of antigenically different surface polysaccharides (5, 9, 10).

The aim of the present study was to investigate intrastrain variation in *B. fragilis* populations which were homogeneous with respect to encapsulation. The stability of expression of individual polysaccharide epitopes within *B. fragilis* populations which are noncapsulate by light microscopy (EDL enriched) was therefore examined.

We now report that populations already enriched for capsule type can also be enriched for expression of individual surface polysaccharide epitopes in both broth and plate cultures.

MATERIALS AND METHODS

Bacterial strains and culture methods. The strains used were *B. fragilis* NCTC 9343 (National Collection of Type Cultures, London, United Kingdom), LS66 and LS54 (clinical isolates from an abdominal abscess and a perianal abscess respectively; Craigavon Area Hospital, Northern Ireland, United Kingdom) (17), and JC17 (clinical isolate from an abscess; Belfast City Hospital, Northern Ireland, United Kingdom). All strains were enriched on Percoll density gradients for populations which were noncapsulate by light microscopy. Bacteria were grown in defined minimal medium (DM) broth or on DM plates (28) in an anaerobic cabinet (Mk. III anaerobic cabinet; 80% N_2, 10% CO_2, and 10% H_2; Don Whitley Scientific, Shipley, United Kingdom). Identification was confirmed with the API20A (Biomérieux, Marcy L'Etoile, France) system.

Production and characterization of polyclonal antisera and MAbs. Polyclonal antiserum specific for *B. fragilis* NCTC 9343 common antigen was produced as previously described (17). Polyclonal antiserum specific for *B. fragilis* NCTC 9344 common antigen was the kind gift of I. Poxton, University of Edinburgh. MAb

* Corresponding author. Mailing address: Department of Microbiology and Immunobiology, School of Medicine, Queen's University of Belfast, Grosvenor Rd., Belfast BT12 6BN, United Kingdom. Phone: 44 (0)1232 240503. Fax: 44 (0)1232 439181. E-mail: s.patrick@qub.ac.uk.

production and initial characterization of some of the MAbs are detailed in reference 5. MAb QUBF 12 does not cross-react with *E. coli* but does cross-react with *Bacteroides thetaiotaomicron*, *B. vulgatus*, and *B. ovatus*. The other MAbs do not cross-react with *E. coli*, *B. vulgatus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, or *Porphyromonas gingivalis*.

Where necessary, hybridoma culture supernatants were concentrated in Vivaspin 15 concentrator filter units (Vivascience Ltd., Lincoln, United Kingdom), and suitable working dilutions in 0.01 M phosphate-buffered saline (PBS; 0.15 M NaCl, 0.0075 M Na_2HPO_4, 0.0025 M $NaH_2PO_4 \cdot 2H_2O$ [pH 7.4]) were determined empirically. Sodium dodecyl sulfate-PAGE was performed on vertical slab gels (8%), which were immunoblotted as previously described (5), using a crude aqueous phenol extract prepared as described by Poxton and Brown (18).

Separation and enrichment of bacterial populations. Bacterial populations which were noncapsulate by light microscopy were enriched by subculture from the 60 to 80% interface layer of Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradients after centrifugation as previously described (11). Encapsulation was monitored by eosin-carbol fuchsin negative staining for light microscopy (1). When required, these populations were further enriched by using immunomagnetic beads as detailed below. Dynal M-280 magnetic beads precoated with sheep anti-mouse immunoglobulin [Dynal (UK) Ltd., Merseyside, United Kingdom] were used as described by Patrick and Larkin (11). In brief, the beads were washed by placing the tube containing the beads in a magnetic particle concentrator and removing the PBS by pipette. Washed beads were suspended in hybridoma culture supernatant containing the relevant antibody, incubated at room temperature with gentle rocking for 24 h, and again washed in PBS. The following steps were all carried out inside an anaerobic cabinet to ensure bacterial viability. The beads were incubated with bacterial suspension (10^8 CFU/ml) in DM plus 0.02% (vol/vol) Tween 20 (Sigma Chemical Co. Ltd., Poole, United Kingdom) with gentle rocking for 2 min and washed three times in DM. The beads were then inoculated into DM and incubated at 37°C in the anaerobic cabinet until bacterial growth was visible.

Immunofluorescence microscopy. Bacterial suspensions in PBS were applied to multiwell microscope slides, dried at 37°C, and fixed either in methanol at −20°C for 10 min or in paraformaldehyde (4% [wt/vol] in 3× PBS [390 mM NaCl, 30 mM Na_2HPO_4, 30 mM $NaH_2PO_4 \cdot 2H_2O$ {pH 7.2}) at 4°C for 30 min (20). For single labelling, the bacteria were then reacted with a suitable dilution of concentrated mouse MAb hybridoma supernatant followed by goat anti-mouse immunoglobulin G (heavy and light chain) conjugated to fluorescein isothiocyanate (FITC; Sigma) as previously described (11). For dual labelling, after incubation of the slides with MAb, they were washed, incubated with anti-*B. fragilis* common antigen polyclonal rabbit antiserum (17), washed, and incubated with sheep anti-rabbit FITC and goat anti-mouse tetramethyl rhodamine isothiocyanate (TRITC; Sigma) before a final wash (11). Slides were examined with a Leitz fluorescence microscope. An estimate of the proportion of bacteria fluorescing was obtained either by comparing FITC-labelled bacteria with bright-field phase-contrast view or by comparing populations dual labelled with anti-rabbit polyclonal antiserum and an anti-rabbit FITC conjugate and with mouse MAb and an anti-mouse TRITC conjugate. All estimations of percentage labelling involved counting a minimum of 100 bacterial cells per well.

Colony lifts and immunoreaction. For colony lifts, total viable counts were carried out after serial 10-fold dilution in Ringer's solution (25% [wt/vol]) with cysteine (0.05%) and spread plating onto DM agar. Unless stated otherwise, the plates were incubated for 48 h. Plates with approximately 150 or fewer colonies were chosen and processed as follows. Discs of nitrocellulose (Millipore UK Ltd., Watford, United Kingdom) were gently applied to the agar plate and lifted once the whole nitrocellulose disc appeared to be wet. A maximum of three lifts were carried out on each plate. For sterile lifts, the nitrocellulose discs were autoclaved prior to use. The nitrocellulose discs were then air dried at room temperature (approximately 22°C) under aerobic conditions for a minimum of 1 h, blocked with dried milk (5% [wt/vol]; Marvel, Chivers Ireland Ltd., Dublin, Ireland) in PBS with Tween 20 (0.05%) for 1 h at 37°C, washed five times rapidly in PBS-Tween followed by five 5-min washes, and either allowed to dry and stored at room temperature in sealed polythene bags for later immunoreaction or used immediately. For immunoreaction, the discs were incubated with the appropriate mouse MAb diluted in PBS for 1 h at 37°C with gentle rocking and washed rapidly five times in PBS-Tween followed by 5-min washes with gentle rocking. The discs were then incubated for 1 h as described above with goat anti-mouse alkaline phosphatase conjugate (Bio-Rad Laboratories Ltd., Hemel Hempsted, United Kingdom). The discs were washed as before except that the final wash was carried out in Tris buffer (50 mM Tris HCl [pH 9.4]) prior to incubation in the substrate (Bio-Rad alkaline phosphatase substrate kit; *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate) according to the manufacturer's instructions. Once the substrate color had reached sufficient intensity, the reaction was stopped by immersion in distilled water and the nitrocellulose was blotted dry with filter paper. Control reactions in which the MAb was replaced with PBS were also processed. Colonies from the control reaction appeared light grey in color, which may have been due to background bacterial alkaline phosphatase activity; however, positive colonies were obviously bright purple-blue. Positive colonies were counted and compared with the initial colony count of the agar plate.

Protocol for determination of antigen expression in broth and plate cultures. For estimates of the proportion of bacteria expressing an epitope in broth culture, four replicate 100-ml volumes were inoculated and incubated at 37°C in the anaerobic cabinet until they had reached an optical density at 600 nm of 0.3 (equivalent to approximately 3×10^8 CFU/ml). The proportion of bacteria within each broth which labelled with a given MAb was estimated by immunofluorescence microscopic examination of 20 separate-microscope slide wells for each broth. A minimum of 100 bacteria were counted in each of the 20 wells.

A 10-fold dilution series was carried out for each of the replicate broth cultures and three replicate spread plates prepared for each dilution. Three colony lifts were taken from each of two replicate spread plates for antibody reaction. Twenty colonies were picked from each of the remaining spread plates (80 colonies in total), and the number of bacteria which labelled with a given MAb was estimated for each colony by immunofluorescence microscopy. Analyses of variance indicated that there was no significant difference between the replicate broth cultures (95% probability).

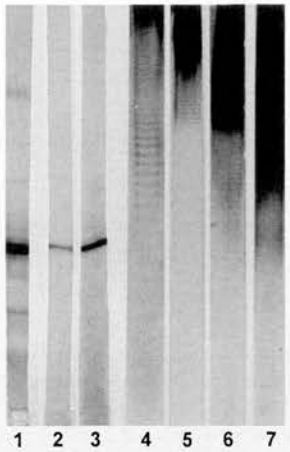

FIG. 1. Immunoblots of hot phenol-water extracts from *B. fragilis* NCTC 9343 after PAGE reacted with rabbit anti-*B. fragilis* NCTC 9343 common antigen antiserum (track 1), rabbit anti-*B. fragilis* NCTC 9344 common antigen antiserum (track 2), and MAbs QUBF 12 (track 3), QUBF 5 (track 4), QUBF 6 (track 5), QUBF 7 (track 6), and QUBF 8 (track 7).

RESULTS

Epitope expression in broth culture. Immunomagnetic bead separation and broth enrichment with MAbs QUBF 6 and 7, specific for high-molecular-mass polysaccharide with an associated fine ladder pattern, and MAb QUBF 12, which recognizes an antigen similar in molecular mass to the *B. fragilis* common antigen (Fig. 1), was successful as assessed by immunofluorescence microscopy. The proportions of bacteria within a population which expressed the various epitopes could be increased from 15 to 76% (QUBF 6), from 21 to 74%, (QUBF 7), and from 22 to 56% (QUBF 12) with two subsequent immunomagnetic bead separations and broth enrichments. The degree of enrichment obtained on different occasions was not, however, always consistent. On a separate occasion, the QUBF 6 epitope was increased only to 32% after two enrichment steps.

Stability of QUBF 6 and 7 epitope expression in *B. fragilis* noncapsulate populations. The reactivity of MAbs QUBF 6 and 7 which are specific for high-molecular-mass polysaccharide with an associated fine ladder pattern was examined on pre- and post-immunomagnetic bead-enriched cultures of *B. fragilis* NCTC 9343. The proportion of bacteria labelling within replicate broth cultures was determined by immunofluorescence microscopy, and the proportion of colonies which labelled on spread plates prepared from the broth cultures was determined by colony blotting. The proportion of colonies which were positive was similar to that of the proportion of individual bacterial cells positive by immunofluorescence microscopy in the original broth culture (Table 1). Similar results were obtained for three other strains of *B. fragilis* (Table 2),

TABLE 1. Reactivity of broth cultures and colonies of noncapsulate *B. fragilis* NCTC 9343 with MAbs QUBF 6 and 7 before and after immunomagnetic bead enrichment

MAb	Culture type	% of bacterial cells in broth culture labelled by immunofluorescence microscopy (mean ± SE)	% of colonies labelled by immunoreaction after growth of broth culture on agar plates (mean ± SE)	% of positive[a] colonies on agar plate as determined by immunofluorescence microscopy of randomly selected colonies (mean ± SE)
QUBF 6	Preenriched	15 ± 2	14 ± 1	0
	Postenriched	32 ± 1	30 ± 4	29 ± 3
QUBF 7	Preenriched	21 ± 2	20 ± 3	15 ± 2
	Postenriched	74 ± 2	51 ± 4	62 ± 3

[a] 94% or more bacterial cells positive.

although the proportion of bacteria that labelled was not the same for different strains labelled with the same MAb. The failure to detect any positive colonies by immunofluorescence microscopy after random selection of 20 colonies per plate, where it was estimated by colony lifts that 14% of the colonies were positive, is probably due to the small sample size.

The proportion of bacteria labelling within the individual colonies was assessed by immunofluorescence and phase-contrast microscopy. Either 94% or more of the cells in a colony were positive by immunofluorescence or less than 7% of the bacteria labelled (Fig. 2 and Table 3). The proportion of colonies showing 94% or greater labelling as estimated by immunofluorescence microscopy, scored as positive colonies, was comparable with the proportion of immunoreactive colonies on the nitrocellulose lifts and in the original broth culture (Table 1). To determine the relationship between colonies positive by blotting and the proportion of the population labelled by immunofluorescence microscopy, lifts were carried out with sterile nitrocellulose and the plates were reincubated for 24 h to allow the colonies to regrow. Colonies were then picked off and analyzed by immunofluorescence microscopy. Colonies with 94% or more bacteria positive by immunofluorescence were also positive by immunoblotting. Immunoblot-negative colonies related to those with 7% or less of the populations labelling by immunofluorescence microscopy.

Examination of the proportion of bacteria labelling in immunoblot-negative colonies indicated that this was consistent in the four replicate broth cultures inoculated in parallel. The proportion of bacteria within these immunoblot-negative colonies was, with the exception of strain LS66, considerably less than the proportion which labelled in the original broth culture. An increase in the length of incubation of the colonies from 48 to 144 h and concomitant increase in colony size did not increase the proportion of the bacteria within the colony which were labelled. With the exception of strain LS66, there was a clear difference in the degree of labelling in immunoblot-negative colonies between QUBF 6 and QUBF 7. Continuous daily subculture of colonies of strain NCTC 9343 in DM broth for 5 days resulted in a maintenance of the level of expression of the epitopes at either 94% or greater or 7% or less for both QUBF 6 and QUBF 7.

Cross-reactivity of epitope-enriched populations with other *B. fragilis*-specific MAbs. Populations enriched using immunomagnetic beads coated with either QUBF 6 (specific for high-molecular-mass polysaccharide) or QUBF 12 (specific for a band similar in molecular mass to the common antigen) and subcultured in DM broth were examined for reactivity with other MAbs by immunofluorescence microscopy (Table 4), as were populations enriched by subculture of colonies immunoblot positive for QUBF 6 and 7 (specific for high-molecular-mass polysaccharide) into DM broth (Table 5). Reactivity of the enriched populations with MAbs showed that with none of the MAbs tested was the proportion of bacteria labelled as great as that labelled with the MAb for which the population had been enriched.

QUBF 6 and 7 immunoblot-positive colonies were subcultured into DM broth and examined for reactivity with QUBF 5 and other MAbs specific for high-molecular-mass polysaccharides (Table 5). With the exception of the reactivity of MAb QUBF 7 with QUBF 6-positive cultures, only a very small proportion of the bacteria reacted.

DISCUSSION

The results clearly illustrate intrastrain antigenic variation of the surface polysaccharides of *B. fragilis*. The epitopes recognized by the MAbs are present on distinct bacterial cells and are detectable on highly variable proportions of bacterial cells within individual natural populations as determined by immunofluorescence and immunoelectron microscopy (9).

The results show conclusively that the proportion of bacterial cells within a population which express these variable polysaccharides can be enriched by using immunomagnetic beads coated with the relevant MAb, followed by broth culture. The lack of reproducibility in the level of enrichment obtained by using immunomagnetic beads on different occasions was probably due to varying numbers of outer membrane vesicles (14) attaching to the magnetic bead bound antibodies and thus preventing bacterial attachment.

The proportion of bacteria which were labelled with MAbs QUBF 6 and 7, which both label polysaccharide in the high-molecular-mass region and an associated fine ladder pattern, was different within the same population of a given strain (Tables 1 and 2). For example, in two strains 50% or more of the bacteria were labelled with MAb QUBF 7, but very few or none of the cells labelled with MAb QUBF 6. This finding

TABLE 2. Immunoreactivity of noncapsulate *B. fragilis* strains with MAbs QUBF 6 and 7

MAb	Strain	% of bacterial cells in broth culture labelled by immunofluorescence microscopy (mean ± SE)	% of colonies labelled by immunoreaction after growth of broth culture on agar plates (mean ± SE)
QUBF 6	NCTC 9343	15 ± 2	14 ± 1
	LS54	6 ± 2	1
	LS66	22 ± 3	19
	JC17	0	0
QUBF 7	NCTC 9343	21 ± 2	20 ± 3
	LS54	50 ± 6	53 ± 5
	LS66	<1	0
	JC17	50 ± 1	60

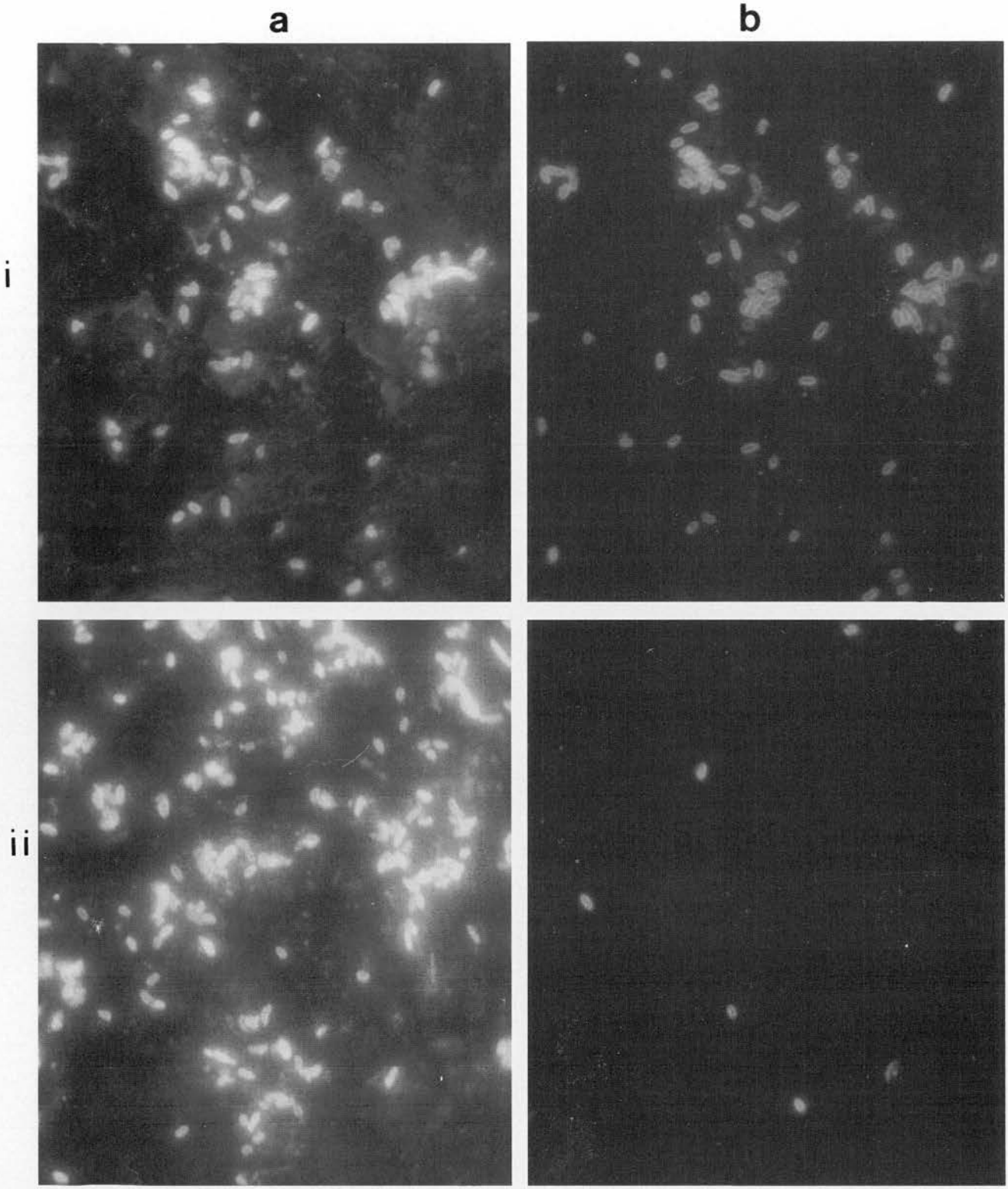

FIG. 2. Light micrographs of *B. fragilis* NCTC 9343, prepared from single colonies, immunolabelled with both mouse MAb plus anti-mouse TRITC conjugate and rabbit anti-*B. fragilis* polyclonal antiserum plus anti-rabbit FITC conjugate viewed (100× objective) with fluorescein filters (a) and the same field viewed with rhodamine filters (b). (i) Colony labelled with MAb QUBF 6 in which 95% or more of the bacteria are labelled; (ii) another colony labelled with MAb QUBF 6 in which only a small proportion of the total bacterial population is labelled.

TABLE 3. Proportion of bacteria within colonies, either positive or negative by colony blotting, which label with MAbs QUBF 6 and 7 by immunofluorescence microscopy

B. fragilis strain	% of bacteria (mean ± SE)[a]			
	Positive colony		Negative colony	
	QUBF 6	QUBF 7	QUBF 6	QUBF 7
NCTC 9343	97 ± 1	94 ± 2	3 ± 1	7 ± 1
LS54	ND	99 ± 1	<1	5 ± 2
LS66	99 ± 0.5	ND	<1	<1
JC17	NR	99 ± 1	<1	4 ± 1

[a] ND, not done; NR, no reaction.

suggests that although these polysaccharides appear to be immunochemically similar after PAGE and immunoblotting, they are antigenically different and that the number of bacteria within a population which express these epitopes varies from strain to strain. The level of expression of MAbs QUBF 6 and 7 within individual colonies was, however, strikingly reproducible. Examination of individual colonies, picked from an agar plate, showed either a high proportion (>94%) or low proportion (<7%) of bacteria labelled for all the strains examined. These proportions were maintained on subculture of individual colonies into broth. This is similar to the phenomenon of enrichment for expression of different capsule types after Percoll density gradient centrifugation (9), which is also maintained on subculture into broth. The relationship between the number of colonies positive for a given epitope and the proportion of bacteria that were labelled in the broth culture from which the colonies were derived was also consistent. It is likely that colonies with a low proportion of bacteria expressing the epitope were derived from a cell which did not initially carry the epitope and that some type of switching mechanism has generated the variants. The rate of switching appeared to occur at a constant rate for the epitopes examined, as the estimated level of expression remained constant for different colonies and between experiments carried out at different times. The proportion of bacteria expressing an epitope was maintained when colonies were subcultured into broth continuously for up to 5 days. This finding suggests that either the switching mechanism observed within the colonies does not function in the relatively homogeneous environment of the broth culture or it occurs at a much slower rate. Given the apparent constant rate of change in the colonies, it is likely that there is an underlying genetic basis to the mechanism that generates the variation. This is a well-documented phenomenon in other pathogenic bacteria (24). It remains to be determined if there is also an environmental influence on this type of variation. Possibilities include external influences such as nutrient availability or changes in the redox potential in the microenvironment. Another possibility is that bacterial signalling molecules such as for example the *N*-acyl homoserine lactones (2) are involved.

TABLE 4. Reactivity of noncapsulate *B. fragilis* NCTC 9343 broth cultures with different MAbs after enrichment for epitope expression by use of immunomagnetic beads

Reactive MAb	% of bacteria labelled by immunofluorescence microscopy (mean ± SE)	
	QUBF 6 enriched	QUBF 12 enriched
QUBF 6	76 ± 3	42 ± 3
QUBF 12	32 ± 2	56
QUBF 5	7 ± 3	8 ± 2

TABLE 5. Proportion of *B. fragilis* NCTC 9343 bacteria in broth culture, derived from colonies positive for either QUBF 6 or 7 by colony blotting, which are MAb reactive by immunofluorescence microscopy

Reactive MAb	% of bacteria labelled by immunofluorescence microscopy (mean ± SE)	
	Enriched from QUBF 6-positive colony	Enriched from QUBF 7-positive colony
QUBF 6	98	1.5
QUBF 7	10	99
QUBF 8	1.5	1
QUBF 12	0	<1
QUBF 5	1	0

These have been well characterized in other gram-negative bacteria, although to date no evidence has been presented for the production of these or similar molecules in *Bacteroides* spp. An environmental influence could perhaps explain the differences observed between the colonies and the broth culture.

The lack of cross-reactivity of populations enriched from colonies in which 98% or more labelled with a given MAb with other MAbs (Table 5) suggests that enrichment for one polysaccharide epitope does not result in coenrichment for a second epitope. Whether this represents exclusive production of one antigenic type or the masking of one by another remains to be determined. The patterns generated by PAGE and immunoblotting, illustrated in Fig. 1, suggest that *B. fragilis* may express three distinct components extractable by the hot phenol-water method. It is likely that these three components are the smooth lipopolysaccharide (QUBF 5), high-molecular-mass polysaccharide (QUBF 6 to 8), and common antigen (QUBF 12) described by Poxton and Brown (18). Furthermore, the lack of cross-reactivity of the populations enriched from QUBF 6- or 7-positive colonies with the other MAbs examined (Table 5) indicates that there are at least three antigenic types of high-molecular-mass polysaccharide with an associated ladder pattern. A further two MAbs, which also did not cross-react with populations enriched from QUBF 6- or 7-positive colonies, have a PAGE pattern indicative of high-molecular-mass polysaccharide but lacking the ladder pattern (unpublished data).

The precise nature of the biochemical differences which generate these different patterns of labelling are unknown. It is possible for polysaccharides to be biochemically similar in terms of components (e.g., sugar moieties) but for a wide variety of antigenic variation to be generated by alteration of either the linkage of the substituent moieties, their chemical substitution, or both. Antigenic variation generated by these means is well documented in the polysaccharides of other pathogenic bacteria such as *Haemophilus influenzae*, *E. coli*, and *Neisseria meningitidis* (12). As yet, no chemical analyses have been carried out on the antigens described in this report. Their relationship with the chemically characterized polysaccharides A and B described by Pantosti and coauthors (7, 8) is therefore unknown. Polysaccharides A and B were obtained from the fraction referred to as capsular polysaccharide (CP) by boiling in 5% acetic acid for 1 h. MAbs specific for either A or B revealed a broad high molecular mass band after PAGE and immunoblotting of the CP fraction. After acetic acid hydrolysis, a narrower band in the highest-molecular-mass region of the broad band was visible for both fractions A and B and their respective MAbs. It therefore appears that polysaccharides A and B also give a similar pattern after PAGE and immunoblotting. A ladder pattern associated with the broad band was detected in the CP fraction by using polyclonal an-

tiserum. These authors suggested that this ladder pattern represented the ladder pattern described by ourselves in the small capsule subpopulation enriched from strain NCTC 9343 by Percoll density gradient centrifugation (23) and also the ladder pattern interpreted by Poxton and Brown as a possible O antigen (18). The ladder pattern illustrated by Poxton and Brown is similar to that detected by our MAb QUBF 5 (Fig. 1) and has wider-spaced bands than that illustrated by Pantosti and coauthors (8). Their ladder pattern is, however, similar in appearance to that observed in the small-capsule population (5, 23). As strain NCTC 9343 normally contains a mixture of large-capsule, small-capsule, and noncapsulate bacteria, it is likely that polysaccharides A and B were extracted from a population with a mixture of types of capsule as well as antigenic types.

The biological activity of our phenol-water extracts was not investigated. Delahooke and coauthors (3), however, compared the immunomodulatory activity of polysaccharide material extracted from *B. fragilis* by different methods and reported a high level of in vitro biological activity in phenol-water extracts. Material extracted from *B. fragilis* NCTC 9343 grown in the same defined medium as we used (28) was 10 times more active in the *Limulus* amoebocyte lysate assay than material obtained from *E. coli* O18:K$^-$. These authors acknowledge that their *B. fragilis* extracts were probably heterogeneous both in M_r and molecular composition. It would be interesting to relate this biological activity to the potential variety of components identified in the present study.

The capacity for antigenic variation could clearly be advantageous to the survival of *B. fragilis* in both its pathogenic mode of existence and in its role as a member of the normal intestinal microbiota. These results also have implications for any studies of the virulence of *B. fragilis* and the chemical nature of these polysaccharides. Inter- and intrastain variation can be clearly observed not only in recent clinical isolates but also directly in pus samples with MAb labelling and immunofluorescence microscopy (17, 25, 26). Studies of the immunological diversity of *B. fragilis* which rely only on whole-cell dot immunobinding or enzyme-linked immunosorbent assay (7) will not detect this within-strain diversity. The intrastrain variation will result in titers in such assays which reflect the proportion of the bacteria within the population which are expressing the epitope. This intrinsic variability of the surface polysaccharides will also need to be taken into account in the production of potential vaccines and immunodiagnostic tests based on polysaccharides.

In conclusion, surface polysaccharides of noncapsulate *B. fragilis* are antigenically highly variable within individual strains. Different antigenic types can be detected on different bacterial cells within an individual population of a given strain. The proportion of bacterial cells carrying any given epitope will be variable depending on the strain and how it has been cultured in the laboratory. Production of the same polysaccharide by all the bacteria within a strain of *B. fragilis* is unlikely and cannot be assumed.

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Search for enterotoxin gene in Bacteroides fragilis strains isolated from clinical specimens in Poland, Great Britain, the Netherlands and France

Mirosław Łuczak[1], Piotr Obuch-Woszczatyński[1], Hanna Pituch[1], Piotr Leszczyński[1], Gayane Martirosian[1], Sheila Patrick[2], Ian Poxton[3], Rob G. F. Wintermans[5], Luc Dubreuil[4], Felicja Meisel-Mikołajczyk[1]

[1] Department of Medical Microbiology, Center of Biostructure Research, Medical University of Warsaw, Poland
[2] Department of Microbiology and Immunobiology, School of Medicine, Queens University of Belfast, Great Britain
[3] Department of Medical Microbiology, University of Edinburgh, Medical School, Great Britain
[4] Franciscus Ziekenhuis, Roosendaal, the Netherlands
[5] Bacteriologie et Virology Faculte des Sciences Pharmaceutique et Biologique, Universite de Lille, France

key words: bacteroides fragilis, enterotoxin fragilysine, PCR

SUMMARY

Background: *Bacteroides fragilis is a member of normal human flora and well known pathogenic agent. This bacterium produces many virulence factors. In 1984 new virulence factor – enterotoxin was described. The aim of the study was to search for enterotoxin gene in B. fragilis strains isolated from clinical specimens.*
Material and Methods: *Strains isolated in Poland, Great Britain, France and the Netherlands were cultured on BBE medium. For DNA isolation Genomic DNA PREP PLUS isolation kit manufactured by A&A Biotechnology (Poland) was used. In order to detect enterotoxin (fragilysin) gene, polymerase chain reaction (PCR) was applied utilizing the following primers: 404 (GAG CGG AAG ACG GTG TAT GTG ATT TGT) and 407 (TGC TCA GCG CCC AGT ATA TGA CCT AGT). DNA obtained from bacterial cells was amplified in thermocycler Techne. The amplification products were detected by the electrophoresis in 1% agarose gel.*
Results: *Among 65 investigated B. fragilis strains, the enterotoxin gene was detected in DNA isolated from 12 strains.*
Conclusion: *The enterotoxin producing B. fragilis strains were detected among strains isolated from different clinical specimens in Poland, Great Britain, the Netherlands and France.*

BACKGROUND

Bacteroides fragilis, a member of the B. fragilis group, is a Gram–negative asporulating encapsulated anaerobic rod. This bacterium is bile-stimulated and extremely saccharolytic. It inhabits the colon of healthy animals and humans. Bacteroides fragilis is the most often isolated anaerobe from clinical specimens as the etiological agent of endogenic suppurative soft tissue infections, abscess or bacteremia [1]. The most important virulence factors of B. fragilis are: capsule, lipopolysaccharide (LPS), outer membrane proteine (OMP), pili, short-chain fatty acids [2]. The role in pathology and mechanisms of action of those virulence factors were studied in different laboratories. The structure and biological activity of

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Correspondence address: Felicja Meisel-Mikołajczyk MD PhD, Department of Medical Microbiology, Center of Biostructure Research, Medical University of Warsaw, 5 Chałubińskiego St., 02-004 Warsaw, Poland, e-mail: fmikolaj@ib.amwaw.edu.pl

the capsule comprised the impact on the abscess formation, the antiphagocytic activity and the mechanisms of adhesion [3]. The structure and biological activity of B. fragilis lipopolysaccharide was described by many authors [4–8]. In 1984 the acute form of enteric disease characterized by profuse diarrhoea, loss of appetite and depression with a high mortality rate was observed in 24–48 h newborn lambs. None of the well established diarrhoeagenic infectious agents appeared to be the cause. Because of the acute nature of the clinical picture of infection, the authors postulated that the cause of infection is a bacterium with a classic enterotoxin secretory activity. Myers et al [9] isolated B. fragilis for the first time and described its enterotoxin activity. Enterotoxin activity was tested through the observation of accumulation of fluid in the lamb intestinal loop (test LIL) [10]. The association of diarrhoea in foals and young pigs with enterotoxigenic B. fragilis (ETBF) was also established (Myers et al 1987) [11,12]. The acceptance of B. fragilis as a new diarrhoeic causative agent in animals led directly to the questions whether it is possible to isolate the enterotoxigenic B. fragilis strains from the human gut flora and whether these could be the diarrhoeic agent. The observations by Meyers et al provided a positive response [13]. The study of Shoop et al [14] has shown the presence of ETBF in municipal sewage. The observations of Weikel et al [15] have shown that the enterotoxin can be tested on human colonic epithelial cells HT29/C1. Van Tassel et al was the first to isolate purified enterotoxin and describe its structure [16]. Very soon in different countries, strains of B. fragilis ETBF were detected: USA [17], France [18], Italy [19], the Netherlands [20], Japan [21], Poland [22–25], Bangladesh [26], Sweden [27]. As isolation of enterotoxin producing strains was described by different authors, it appeared to be worthwhile to test the strains isolated in different countries.

The aim of the present study was to test the DNA from the strains isolated in different countries using the PCR methods to find the strains possessing the enterotoxin gene.

MATERIAL AND METHODS

Strains

Sixty five Bacteroides fragilis strains isolated from human extraintestinal sources in different countries (tab I.) were used in the experiment.

1) Out of 65 strains 18 were isolated in Poland: 1/B, 2/B, 3/B, 5/B, 6/B, 7/B, 8/B, 9/B, 10/B, 11/B, 12/B, 13/B, 14/B, 15/B, 17/B, 18/B, 19/B, 22/B.
2) Seventeen were isolated in Belfast UK: JC6, JC15, JC17, JC19, LS27, LS54, LS66, LS67, BE1, BE3, GNAB4, GNAB82, GNAB92, DK2, DK5, DK9, DK10.
3) Ten were isolated in Edinburgh UK: 3367, 3422, 3505, 3718, 3822, 3823, 3837, 3845, 3851, 3871.
4) Ten strains were isolated in Rosendaal NL: 1H, 2H, 3H, 4H, 5H, 6H, 7H, 8H, 9H, 10H.
5) Ten strains were isolated in Lille France: 9755, 9760, 9762, 9771, 9778, 9701, 9810, 9813, 9816, 9817.

Two reference strains: Bacteroides fragilis NCTC 11295 (ETBF) and IPL 323 (NTBF) were also used as a controls.

Media

Strains were cultured on Columbia agar (Oxoid) enriched with sheep blood, hemin and vitamin K.

DNA extraction

DNA was isolated using Genomic DNA PREP PLUS isolation kit manufactured by A&A Biotechnology (Poland).

PCR reaction

To detect enterotoxin-fragilysine gene polymerase chain reaction was applied.

Following primers were utilised: 404 (GAG CCG AAG ACG GTG TAT GTG ATT TGT) and 407 (TGC TCA GCG CCC AGT ATA TGA CCT AGT). DNA obtained from bacterial cells was amplified in a thermocycler (Techne). The temperature profile was as follows: 1 cycle (4 min. 94°C), 40 cycles (1min. 94°C, 1min. 52°C, 1 min. 74°C).

Electrophoresis

Amplification products were detected by electrophoresis in 1% agarose gel with ethidium bromide added.

RESULTS

Out of 65 strains under investigation 12 appeared to belong to Bacteroides fragilis possessing an enterotoxin gene (Table 1, Figure 1).

Table 1. Results of PCR detected enterotoxin gene in DNA isolated from *B. fragilis* strains.

Country of origine	Number of tested strains	Number of enterotoxin gene positive strains
Poland	18	2
Great Britain I	17	3
Great Britain II	10	2
the Netherlands	10	4
France	10	1
Total	65	12

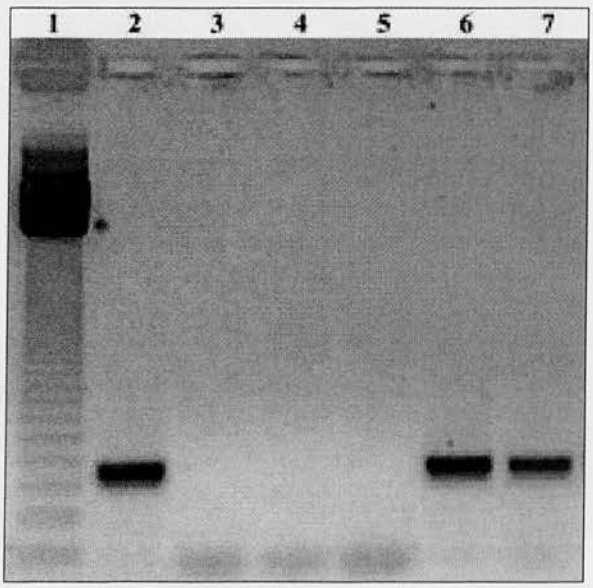

Figure 1. Agarose gel electrophoresis of amplified DNA from selected B. fragilis strains. Lane 1: 123 bp DNA ladder. Lane 2: NCTC 11295 (ETBF) strain. Lane 3: IPL 323 (NTBF) strain. Lane 4: 18/B strain. Lane 5: 3505 strain. Lane 6: 22/B strain. Lane 7: 3823 strain.

DISCUSSION

During the 1990s a relative decrease of anaerobic infections has been reported. Nevertheless, anaerobic bacteria are still a frequent cause of diseases associated with severe morbidity and even mortality. Among anaerobic bacteria the most commonly encountered in the clinical practice are gram-negative rods of the B. fragilis group. In this group, which currently contains 10 species, the most important are B. fragilis and B. thetaiota-omicron. B. fragilis rods are recovered from most intraabdominal infections and infections of other sites below the diaphragm [27]. Although anaerobic infections have been diagnosed in most anatomic sites of the body, the majority arise from the indigenous flora of the mucosa membranes. Since all these organisms are endogenous it is always a predisposing factor for anaerobic infection [27]. The virulence factors and the mechanisms of infections of B. fragilis species have been analysed in detail [3]. Since 1984 [9] a new virulence factor in B. fragilis strains has been observed. Enterotoxin (fragilysine) produced by some of the strains altered the prespective on the importance of those strains [8,11,14]. Very carefully described the structure of the enterotoxin and the mechanisms of its action [15,16] lead to the new trial to answer several questions. The various methods of determining the strains able to produce the enterotoxin: cytotoxicity on HT/29/C1 [22] or PCR [20] helped to establish whether enterotoxin production comprises a new characteristic of some B. fragilis strains or whether those strains were overlooked in earlier studies. In previous studies [20,24] it was shown that before 1984 in Poland and in Holland strains possessing the ability to produce enterotoxin were present. The majority of strains of enterotoxin producing ETBF were isolated from fecal samples of diarrheic people. Some of the publications assure us that the ETBF strains can be isolated out of the extraintestinal sources [21,28]. We have shown previously that the enterotoxin producing ETBF B. fragilis can be isolated together with the toxin producing Clostridium difficile [29]. The interaction of those two infectious agents appeared to provide a new field for experimentation. Because of all those reasons we decided to collect strains from different countries. From earlier reports we know that B. fragilis ETBF strains isolated in Poland were shown to produce highly variable amounts of cytotoxin, and the lack of genetic relationship between the strains was demonstrated by different PCR-mediated DNA typing procedures [30]. It was also shown that B. fragilis enterotoxin gene can appear in different alleles [31].

CONCLUSIONS

The results of the experiments reported in this study provide the first steps to compare the Bacteroides fragilis strains isolated in different countries and to study the role of ETBF strains in different kinds of infections.

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Studies of *Microthrix parvicella in situ* and in laboratory culture: production and use of specific antibodies.
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Studies of *Microthrix parvicella in situ* and in laboratory culture: production and use of specific antibodies

N. Connery*,, A.S. Thompson*,**†, S. Patrick** and M.J. Larkin***

* The QUESTOR Centre & School of Biology and Biochemistry, The Queen's University of Belfast, David Keir Building, Stranmillis Road, Belfast BT9 5AG, United Kingdom

** Department of Microbiology and Immunobiology, School of Medicine, The Queen's University of Belfast, Belfast. BT12 6BN, United Kingdom

† Communicating author (E-mail: *a.s.thompson@qub.ac.uk*)

Abstract Physiological studies on *M. parvicella* have been conducted to determine the rate of growth of this organism in pure culture. The organism displayed a doubling time of 128 days despite its profuse abundance in a local Wastewater Treatment Plant (WWTW). An extensive survey has been ongoing since February 2000 into the extent of *M. parvicella* in the WWTW. A suite of monoclonal and polyclonal antibodies has been developed to detect and quantify *M. parvicella*.

Keywords Activated sludge; bulking; immunofluorescence; *Microthrix parvicella*

Introduction

In the last ten years *M. parvicella* has been recorded as being the most frequently present filamentous micro-organism in bulking sludge in Europe (Eikelboom *et al.*, 1998; Wanner *et al.*, 1998). Its presence in biological nutrient removal (bio-NR) plants is seasonal and appears to be stimulated by temperature and dissolved oxygen (DO) concentration. Despite its profuse abundance in bulking sludge during winter and spring, it is slow growing in pure culture and has an optimum growth temperature of 12°C–15°C (Knoop and Kunst, 1998) making biochemical and physiological studies difficult. In addition, its filamentous nature means that it is difficult to enumerate by conventional direct means.

Immunological methods have been reported for the detection of specific populations of filamentous and non-filamentous bacteria in wastewater systems other than *M. parvicella* (Howgrave-Graham and Steyn, 1988 and Raskin *et al.*, 1998). *M. parvicella* has been detected in sludge using fluorescent *in situ* hybridisation (FISH) (Erhart *et al.*, 1997) but with variable results, and requiring a methodology not suitable for routine analysis within the water industry. The relative ease with which the signal produced using immuno-technologies offers the possibility of quantitative immunofluorescence microscopy (IFM) to complement diagnostic FISH, and provide the basis of quick quantitative and qualitative analyses.

We describe here the results of a long-term survey of municipal WWTWs within Northern Ireland, a number of physiological growth experiments on pure cultures of *M. parvicella* and the production and use of a range of antibodies specific to *M. parvicella*.

Methods

Microthrix parvicella RN1 (Rossetti *et al.*, 1997) was obtained from Dr. Valter Tandoi (Water Research Institute, Rome). The growth medium (R2AM) used was a modified form of R2A (Reasoner and Geldreich, 1985), with the additions of (mg per litre medium): $CaCl_2.2H_2O$, 50; NaEDTA, 50; Na_2MoO_4, 20; cycloheximide; 20, NMS Trace Elements (Atlas, 1995), vitamins (Eikelboom, 1968). Supplements to this medium, where mentioned, included centrifuged sludge, filter sterilised (0.2 μm) or autoclaved, filter

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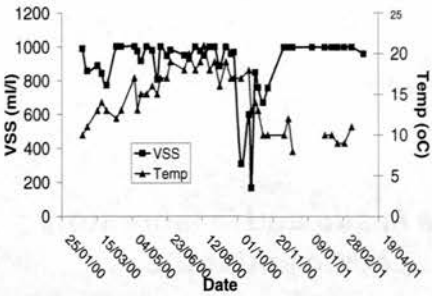

Figure 1 Volume of Suspended Solids (VSS) vs. temperature of sludge

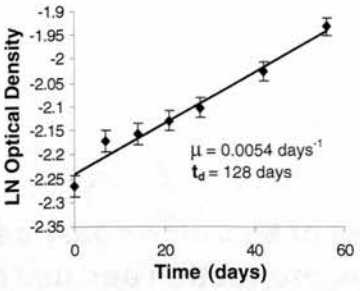

Figure 2 Regression of OD (550 nm) of *M. parvicella* in microtitre plate wells (R2A, $n = 16$)

sterilised spent *M. parvicella* culture supernatant. Cell quantification was by microBCA Protein Assay Kit (Pierce), spectrophotometric analysis (optical density of cultures @550 nm, in microtitre plates).

Polyclonal antibodies and monoclonal antibodies were produced from whole live cells according to previously published methods (Lutton *et al.*, 1991). IFM followed the methods previously reported (Ramage *et al.*, 1998), using methanol fixed cells.

Results and discussion

Survey of WWTWs

A 12-month survey regarding the extent of *M. parvicella* in a municipal WWTW, was carried out from February 2000. This involved the measurement of a number of physical and chemical parameters on a weekly basis (Figure 1). It was found that as temperature increased seasonally, the volume of suspended solids (VSS, in mls) did not reduce, as found elsewhere (Knoop and Kunst (1998), Mamais *et al.* (1998)) with the exception of several samples from October 2000 which were investigated (Connery *et al.*, in preparation). Indeed, this plant has exhibited poor settling continuously during this study, which began in September 1999. Quantities of *M. parvicella* filaments in these samples are seen to follow the trend observed for settling (data not published).

Physiological studies

Despite the abundance of *M. parvicella* in the WWTW sludge, RN1 has been found to grow slowly in pure culture, with a doubling time of 128 days (Figure 2). It has been shown that the culturability of aged *Mycobacterium tuberculosis* cultures is greatly improved when grown in spent culture supernatant (Sun and Zhang, 1999) and the effect of "autoinducing" compounds on Actinomycetes has now been widely reported (see Horinouchi and Beppu, 1992). Some of the work reported here investigated the existence of such a factor (be it an autoregulatory compound or one which is indigenous to sludge), to explain the slow growth observed in pure culture. Typical growth yields can be seen in Table 1 for *M. parvicella*.

The organism was grown on R2AM medium and in R2AM supplemented with sterilised sludge supernatant obtained from the WWTW on 8/3/2000. This sludge sample did not improve the growth yield beyond that of the non-supplemented medium, R2AM. Figure 3

Table 1 Growth yields expressed as total protein of *M. parvicella* in sludge-supplemented media. ($n = 5$, $p<0.05$)

Medium A	Medium B	Medium C
Sludge supernatant	Sludge supernatant	–
Glucose, Pyruvate	–	Glucose, Pyruvate
R2AM base	–	R2AM base
166.0 μg/ml ± 4.09	169.3 μg/ml ± 4.09	175.1 μg/ml ± 6.03

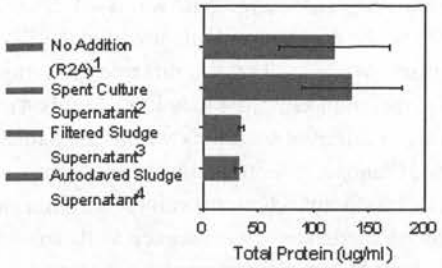

Figure 3 Growth yields for *M. parvicella* grown on R2AM with spent culture supernatant(2) and sludge supernatant from 2/2/00. ($n = 5$, $p<0.05$)

illustrates a similar experiment using a sludge supplement obtained on 2/2/2000 (bars 3 and 4) which indicates that some of these supplements may actually inhibit growth. Growth of *M. parvicella* in R2AM medium supplemented with the supernatant of spent culture was also examined (Figure 3). When grown in the presence of re-used culture medium, growth yields were not significantly increased.

Detection of filaments in sludge by IFM

Polyclonal and monoclonal antibodies have been obtained and characterised (Thompson *et al.*, in preparation). A range of mixed slurries from activated waste treatment systems were examined for *M. parvicella* using conventional means (Eikelboom, 1968) and IFM. All the antibodies described detected natural populations of *M. parvicella* in samples, though some showed brighter fluorescence across a range of samples than others. A cocktail of the monoclonal antibodies was found to be the most reliable method of detecting filaments *in situ*, as the intensity of the autofluorescing background material often reduced the clarity of spe-

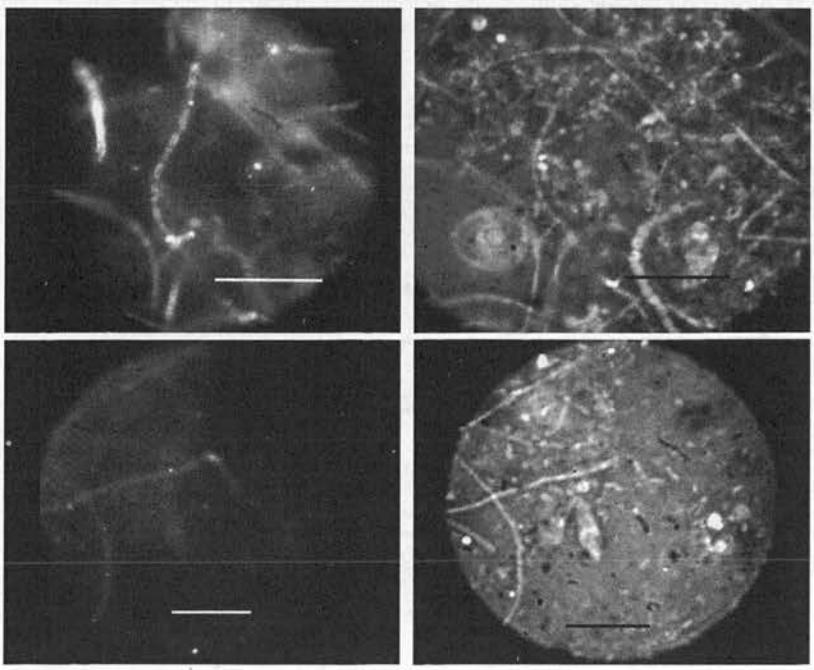

Figure 4 Immunofluorescence by anti-*M. parvicella* rabbit polyclonal antibody of putative *M. parvicella* filaments found in samples taken from Belfast WWTW. Clockwise, starting at top left, fluorescent image of labelled aerated foam sample, phase image of same field of view, phase image of labelled anoxic liquor sample, fluorescent image of same field of view. All views at (approx.) × 800 using a Nikon CP950 digital camera with zoom. Bar represents 20 μm. All samples were methanol fixed, and secondary antibodies were FITC-conjugated

cific antibody staining when only one monoclonal was used. On the basis of IFM analysis, *M. parvicella* was found to be the dominant filament within the WWTW and indeed, it was the dominant microorganism. We also observed different patterns of fluorescence within the same works WWTW at different sampling locations (Figure 4).

Samples were drawn from different locations within the same aerated Carrousel-type system; from a non-aerated ("anoxic") section, mid-aerated and from the final point before discharge to settling tanks (the point where maximum aeration might be expected). The polyclonal antibody showed increasing fluorescence with an increase in aeration. It is possible that this was the result of increased expression of an epitope associated with oxidative metabolism. It is also notable that it was possible to elucidate a fine structure within *M. parvicella* in sludge samples, particularly in brightly fluorescing examples.

Conclusions

M. parvicella has been seen to be present all year round in this WWTW as determined from February 2000 to February 2001. This plant has observed the phenomenon of bulking since February 2000. The growth yield of *M. parvicella* is not improved when grown in the presence of sludge from this plant obtained on either February 2nd or March 8th, 2000. The same may be said for the addition of spent *M. parvicella* culture supernatant. The organism is slow growing with a doubling time of 128 days. A range of specific antibodies has been produced against *M. parvicella* RN1 which have been shown to show varying reactivity against putative *M. parvicella* filaments found in the WWTW.

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91 Bacteroides

Sheila Patrick

The Queen's University of Belfast, Belfast, UK

Bacteroides are present in the faeces and are the predominant bacteria of the open-ended culture system of the human intestinal tract. It has been estimated that *Bacteroides* spp. are one of the major groups of commensals that inhabit the human body. They and other related bacteria are also found in the upper respiratory tract, mouth and urogenital tract of humans and animals. This chapter deals mainly with the *Bacteroides sensu stricto* (Table 1). The oral Gram-negative obligate anaerobes *Porphyromonas*, *Prevotella* and *Fusobacterium* are considered in Chapter 51. Certain *Bacteroides* species, in particular *Bacteroides fragilis* and *B. thetaiotaomicron*, are the strict anaerobes most frequently isolated from clinical infection (Johnson, 1978). Such infections generally arise from the contamination by the commensal microbiota of normally non-colonised body sites. In this sense, *Bacteroides* spp. are opportunistic pathogens.

Table 1 Members of *Bacteroides sensu stricto*[a]

Species	Genome size[b] (Mbp)
B. fragilis (type species)	5.3
B. caccae	
B. distasonis	4.8
B. eggerthii	4.4
B. merdae	
B. ovatus	6.9
B. stercoris	
B. thetaiotaomicron	4.8
B. uniformis	4.6
B. vulgatus	5.1

[a] As defined by Shah and Garbia (1991).
[b] Data from Shaheduzzaman *et al.* (1997).

Though Louis Pasteur is credited with the first cultivation of strictly anaerobic bacteria, it was Veillon and Zuber (1897, 1898) who demonstrated clearly the involvement of Gram-negative non-spore-forming strictly anaerobic bacteria in infections such as brain and lung abscesses, peritonitis, bartholinitis and otitis media. They named the most abundant and frequently observed bacterium in appendicitis and peritonitis *Bacillus* (now *Bacteroides*) *fragilis*. *B. fragilis* is the obligately anaerobic Gram-negative bacterium most frequently isolated from clinical infection and *B. thetaiotaomicron* the second most frequent isolate (Table 2; Duerden, 1980; Brook, 1989; Willis, 1991). These infections include intra-abdominal, vaginal, pilonidal, perianal and brain abscesses and soft-tissue infections. *B. fragilis* is also the most common cause of anaerobic septicaemia, with a potential mortality of up to 19% (Redondo *et al.*, 1995). The risk of *B. fragilis* infection after surgical operations of the lower intestinal tract makes prophylactic antibiotic cover necessary. Before the introduction of antibiotics, the mortality from infection arising particularly from large-bowel perforations was

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Table 2 Incidence of *Bacteroides* spp. in faeces and clinical samples

	[a]Faeces (%)	[a]Clinical samples (%)
B. vulgatus	43–45	2–3
B. thetaiotaomicron	15–29	13–17
B. distasonis	9	3–6
B. fragilis	4–13	63–81
B. ovatus	4	0–7

[a] Compiled from Duerden (1980), Brook (1989), Namavar *et al.* (1989), Willis (1991).

30–100%, for example after rupture of an inflamed appendix. In the 1970s intra-abdominal abscesses were associated with a mortality of up to 30%, largely because the role of anaerobes in these infections was not appreciated and treatment was therefore not given (Tally and Ho, 1987), in spite of the early observations of Veillon and Zuber in the 1890s.

Knowledge and understanding of *B. fragilis* is about to blossom as a result of the provision of funding by the Wellcome Trust for the Sanger Centre (UK) to sequence the complete genome of the *B. fragilis* type strain NCTC 9343 (ATCC 25285) and a partial shotgun sequence of the rifampicin-resistant strain 638R. This sequence information is in the public domain and available for view on the web sites of Wellcome Trust Beowulf Genomics (http://www.beowulf.org.uk/home.htm) and the Sanger Centre (http://www.sanger.ac.uk/Projects/Microbes/).

Classification and Taxonomic Position

The genus *Bacteroides* was at first a repository for any Gram-negative strictly anaerobic non-spore forming bacillus that was neither a *Fusobacterium* nor a *Leptotrichia*, but it has been refined over the years. Many of the more than 50 species listed in the ninth edition of *Bergey's Manual of Systematic Bacteriology* have been assigned to new genera (Table 3). The proposal formally to limit the genus *Bacteroides* to the species listed in Table 1 was made by Shah and Collins (1989). The type species is *Bacteroides fragilis* and, therefore, the 10 species listed in Table 1 are sometimes referred to as the '*B. fragilis* group'. The phylogenetic tree based on whole genome analyses of 7 species of the *B. fragilis* group was reported to be broadly similar to that obtained from 16S rRNA sequences, with the exceptions of *B. thetaiotaomicron* and *B. ovatus*. The sizes of the genomes analysed are included in Table 1 (Shaheduzzaman *et al.*, 1997).

Table 3 Former members of the genus *Bacteroides*

Dichelobacter nodosus
Prevotella
Porphyromonas
Ruminobacter
Campylobacter gracilis
Bacteroides ureolyticus[a]

[a] Designated a member of the *Campylobacteraceae* but not yet re-named.

DNA homology studies indicate that there is a good correlation between DNA homology group and phenotypic characteristics of the designated species (Johnson and Ault, 1978). Although *B. fragilis*, *B. ovatus* and *B. thetaiotaomicron* could each be subdivided into more than one homology group, the phenotypic tests used identified the different homology groups as the relevant species. Of a group of 60 strains of DNA homology group I, for which the source was known, 76.6% were clinical isolates. Of the 11 group II isolates whose source was known, approximately half were from faeces and the other half from infections. More recent analysis of the two DNA homology groups of *B. fragilis* indicate that group I and group II *B. fragilis* can also be distinguished on the basis of ribotyping, analysis of PCR-generated fragment patterns, insertion sequence content (Podglajen *et al.*, 1995) and small-subunit rDNA sequencing (Ruimy *et al.*, 1996). It was noted that *B. fragilis* DNA homology group I lack the gene *cfiA* (also called *ccrA*) which encodes a metallo-β-lactamase (Ambler class B enzyme). This enzyme confers resistance to the majority of β-lactam antibiotics, such as carbapenems, imipenem and meropenem, and is not susceptible to inhibitors such as clavulanic acid, sulbactam and tazobactam. This gene was identified in about 3% of isolates. Only about one-third of the strains examined expressed this β lactamase, however; the gene is silent in the remainder of these strains. The data suggest that DNA homology group II strains relate to the *cfiA*-gene positive group. Interestingly, three insertion sequences (IS4351, 942 and 1186), which provided promoter sequences for the transcription of *cfiA*, were also identified. This will be further considered below. On the other hand group I strains, which represent the majority of clinical isolates, lack the *cfiA* gene but carry the *cepA* gene, which encodes an active-site serine β lactamase, related to the Ambler Class A enzymes. The *B. fragilis* type strain NCTC 9343 (ATCC 25285) and strain 638R (also known as AIP 638R, TM4000, IB101 and 638rfm), which has been used extensively in genetic studies, are members of the DNA homology group I.

Table 4 Position of *Bacteroides* within the Phylum *Bacteroides–Flavobacterium*

Phylum *Bacteroides–Flavobacterium*	
Bacteroides subdivision	Group
Bacteroides	
Bacteroides	fragilis
Prevotella	melaninogenicus–oralis
Porphyromonas	saccharolytic pigmented
Dichelobacter	
Fusobacterium	fusiform
Leptotrichia	
Flavobacterium subdivision	
Flavobacterium	
Cytophoga	
Flexibacter	

In the wider taxonomic picture, on the basis of rRNA sequence comparisons, the *Bacteroides* fall into the *Bacteroides–Flavobacterium* phylum (Table 4; Woese, 1987). Bearing in mind the diverse phenotypes of these two groups of bacteria, this is an unexpected taxonomic association, since *Flavobacterium*, which includes *Cytophaga* and *Flexibacter*, are aerobic. It may be that as more is learned of the characteristics of these two bacterial groups, their similarities will become more apparent. Interestingly, it is thought that this phylum diverged from other eubacteria early in evolutionary terms. This is thought to have occurred well before the divergence of the Gram-positive bacteria from the phylum, which contains the majority of Gram-negative bacteria, such as the enteric bacteria *Escherichia coli* and the pseudomonads (Phylum, Proteobacteria, Purple bacteria). Indeed, the sequences of some antibiotic-resistance genes bear remarkable homology with those of Gram-positive bacteria. This taxonomic divergence may also explain the difficulties that have been encountered in carrying out genetic experiments in *Bacteroides*, since the methods rely heavily on *E. coli* (see below).

For the taxonomic history of the genus *Bacteroides* the reader is referred to Shah and Garbia (1991) and Shah *et al.* (1998).

Identification

The key distinguishing feature of the *Bacteroides* spp. and related anaerobic Gram-negative bacteria is their anaerobic nature, but their ability to tolerate oxygen may be variable (see below). They are non-sporing and are generally non-motile. When grown on anaerobic blood agar, they lack the black pigmentation and fluorescence that may be associated with some of the oral Gram-negative anaerobes, such as *Porphyromonas* or *Prevotella* spp., and they form circular, entire and smooth colonies. They are generally sensitive to metronidazole and resistant to kanamycin, vancomycin and colistin. Haemin and vitamin K are stimulatory for the growth of many *Bacteroides* spp. and they are an essential requirement for some. The major characteristics are detailed in Table 5.

Table 5 Major characteristics of the genus *Bacteroides*

Microscopical
Gram negative,
Non-sporing
Non-motile
Pleomorphic rod-shaped
Cell size 0.5–1.3 × 1.6–11 μm
Components
Principal respiratory quinones: menaquinones (with 10 and/or 11 isoprene units)
Dibasic amino acid of peptidoglycan: *meso*-diaminopimelic acid
DNA G + C ratio: [a]40–48 mol%
Lipid content : sphingolipids
Predominant cell fatty acid: 3-hydroxylated and non-hydroxylated (straight chain saturated, *anteiso*- and *iso*-methyl branched-chain types)
Growth and metabolism
Strictly anaerobic
Bile tolerant
Esculin hydrolysed
Fermentation: saccharolytic, major metabolic end-products acetate and succinate
Metabolic enzymes: enzymes for pentose phosphate pathway e.g. glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and also malate dehydrogenase and glutamate dehydrogenase

Compiled from Shah and Garbia (1991).

[a] Lower figure variously quoted as 39, 40 and 41.

Further details of the current techniques used for the clinical diagnosis of *Bacteroides* spp. are presented below. The reader is also directed to the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993) and relevant chapters in Cowan and Steel's *Manual for the Identification of Medical Bacteria* (Brown *et al.*, 1989; Barrow and Felthan, 1993; Jousimies-Somer *et al.*, 1995). Although it is not yet part of the routine identification of *Bacteroides* spp., there is scope for the extension to clinical practice of identification methods based on 16S rRNA sequences that have been used for the identification of *Bacteroides* in human faeces (e.g. Manz *et al.*, 1996; Bonnett *et al.*, 1999).

Structures

Fimbriae and extracellular polysaccharide capsules are two surface structures that have been studied in detail in *Bacteroides*. The vast majority of structural investigations have centred on *B. fragilis*, which is considered to be the most frequent clinical isolate. Some strains of *B. fragilis* release quantities of outer membrane vesicles that carry with them the surface polysaccharides (Fig. 1). The potential role of these structures in virulence is discussed below.

Polysaccharides

Encapsulating structures have been implicated in resistance to complement-mediated killing, uptake and killing by phagocytes (Reid and Patrick, 1984), and in abscess formation in an animal model (Tzianabos *et al.*, 1993). These aspects are discussed in detail below in relation to virulence.

Capsules are discrete clear areas around negatively-stained *B. fragilis*, *B. ovatus*, *B. vulgatus* and *B. thetaiotaomicron*, but they were not present in five strains of *B. distasonis* (Babb and Cummins, 1978). With the exception of *B. ovatus*, which were all capsulate, 10% or less of the bacteria of other species had capsules. Capsule size varied between strains and also, notably, within populations of a given strain. It is now clear that *B. fragilis* exhibits not only within-strain phase variation in capsule production, but also between- and within-strain antigenic variation of different types of capsules, which continues not to be taken into account. In individual strains of *B. fragilis* it is possible by electron microscopy to identify large (LC) or small capsules (SC) that are both fibrous in appearance but are antigenically different, and bacteria with an encapsulating electron-dense-layer (EDL) adjacent to the outer membrane (Fig. 2) (Patrick and Reid, 1983; Patrick *et al.*, 1986). Bacteria with an EDL are non-capsulate (NC) by light microscopy, but small and large capsules are visible with negative staining. Expression of the different capsular types is inheritable, since populations can be enriched by subculture from different interfaces of Percoll step density gradients. EDL-enriched populations also produce polysaccharide slime (Fig. 3) which shares epitopes with the discrete LC. Observation by microscopy of the populations enriched for the three capsular types with monoclonal antibodies specific for surface polysaccharides shows that non-capsulate bacteria are antigenically different from SC bacteria, but share epitopes with LC bacteria. In addition, immunofluorescent and immunogold labelling for fluorescence and electron microscopy, respectively, reveal antigenic variation in populations

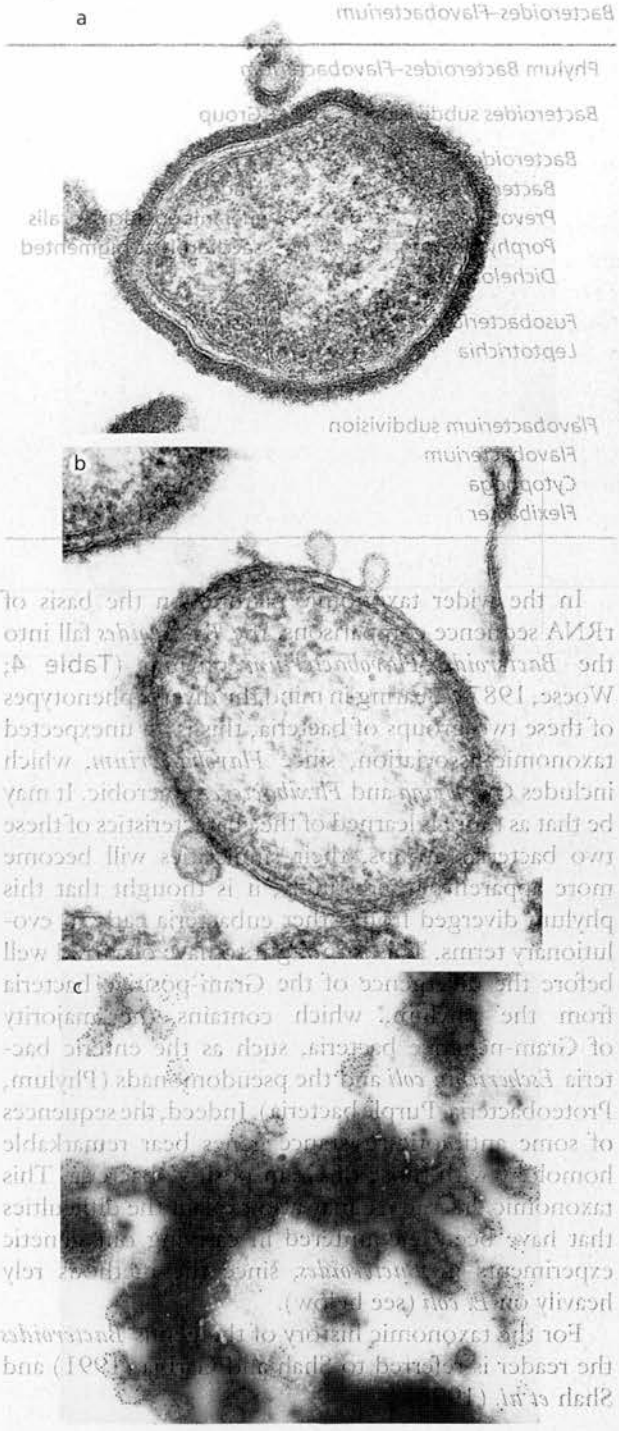


Fig. 1 Transmission electron micrographs of outer-membrane vesicles of *B. fragilis*: (a) Ultrathin section of bacterium (non-capsulate by light microscopy) with an EDL adjacent to the outer membrane. Note the outer membrane vesicle with associated electron dense material. (b) Ultrathin section of bacterium to illustrate vesicle being released from the outer membrane. (c) Negatively stained outer membrane vesicles immunogold-labelled with monoclonal antibody QUBF5. From Lutton *et al.* (1991), with permission.

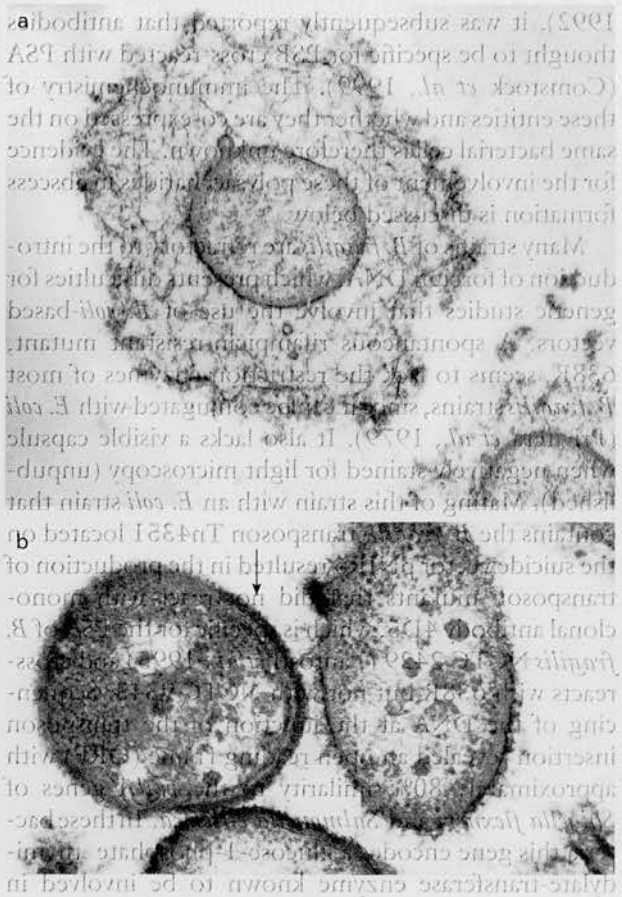


Fig. 2 Transmission electron micrograph of ultrathin sections to illustrate encapsulating structures of *B. fragilis*. (a) Bacterium with large fibrous network, which equates to a LC visible by light microscopy. (b) Bacterium with small fibrous network, which equates to a SC visible by light microscopy (right-hand side) and bacterium with marginal EDL, non-capsulate by light microscopy (left-hand side and arrow).

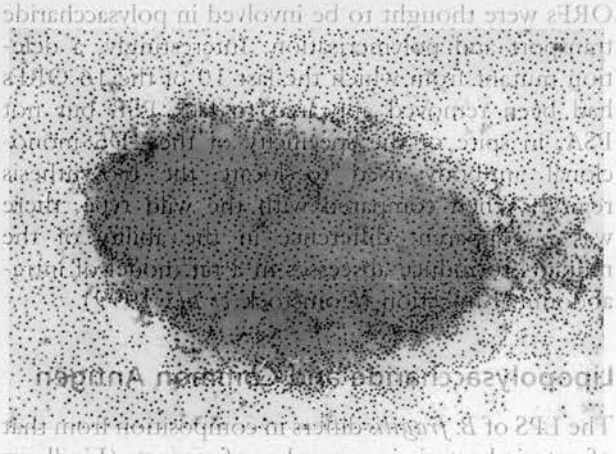


Fig. 3 Transmission electron micrograph of negatively stained bacterium from non-capsulate population of *B. fragilis* immunogold-labelled with monoclonal antibody QUBF7 to illustrate extracellular material. From Lutton *et al.* (1991), with permission.

that appear to be structurally homogeneous (Reid *et al.*, 1985, 1987; Patrick and Lutton, 1990a; Lutton *et al.*, 1991). This phenomenon is observed in recent clinical isolates from a variety of anatomical sites, in isolates from different geographical locations and in culture collection type cultures (Patrick *et al.*, 1995b). If a broth culture with a mixture of epitopes is plated on agar and the resulting colonies are labelled with surface polysaccharide-specific monoclonal antibody and a fluorescent dye and examined by microscopy, two types of colonies are observed; in one of these 90% or more, and in the other 10% or less, of the bacteria express the same epitope. Subculture of single colonies in which the majority of the bacteria express that particular epitope into broth culture, results in enrichment for this epitope, and failure to enrich for other epitopes (Patrick *et al.*, 1999). This strongly suggests that a reversible switching mechanism is in operation.

The genetic mechanism that generates the observed phase and antigenic variation is not known, but multiple mechanisms for the generation of phase and antigenic variation are well characterised in other bacteria (Patrick and Larkin, 1995). Examples which may be relevant to *Bacteroides* include variation in *Neisseria meningitidis* (Jennings *et al.*, 1999) and *Haemophilus influenzae* lipopolysaccharide (LPS).

The precise nature of the biochemical differences that generate the antigenic variation is unknown. It is possible that polysaccharides are biochemically similar in terms of sugar moieties, for example, but for a wide variety of antigenic variation to be generated by alteration of the linkage of the substituent moieties, their chemical substitution or both. Antigenic variation generated by these means is well documented in the polysaccharides of other pathogenic bacteria such as *H. influenzae*, *E. coli* and *N. meningitidis* (Patrick and Larkin, 1995).

Immunochemical analysis of these antigenically variable polysaccharides after polyacrylamide gel electrophoretic (PAGE) separation reveals diffuse bands of high molecular mass. In the non-capsulate, slime-producing populations, associated ladder patterns of lower molecular mass are also observed. This type of banding pattern is characteristic of different chain lengths of heteropolymeric polysaccharides with repeating subunits, each 'step' in the ladder corresponding to the addition of another subunit. It may be that polysaccharides with different chain lengths are more characteristic of slime as opposed to discrete capsular structures. Ladders with a fine step size and a large step size have been observed (Fig. 4). The larger step size resembles the pattern observed with the O antigens of enteric bacteria. The controversy surrounding the O antigen of *B. fragilis* is further discussed below.

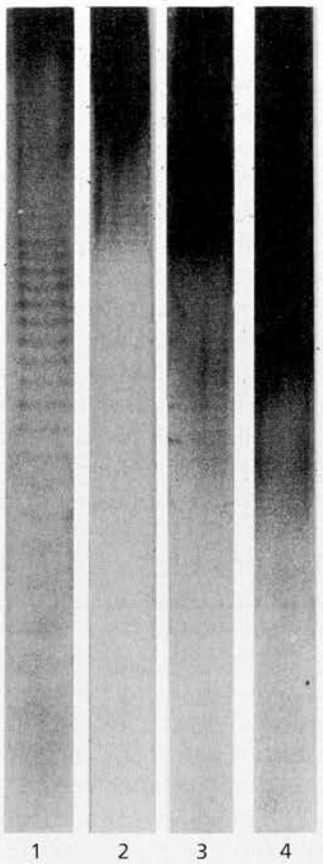


Fig. 4 Immunoblots of aqueous phenol extracts from *B. fragilis* strain NCTC 9343 after PAGE reacted with QUBF5 (track 1), QUBF6 (track 2), QUBF7 (track 3) and QUBF8 (track 4). Note high-molecular-mass material and associated ladder patterns. From Patrick *et al.* (1999), with permission.

Two *B. fragilis* polysaccharides, extracted from populations of strain NCTC 9343 which were not defined for capsule expression by microscopy, have been chemically characterised and designated A and B (Pantosti *et al.*, 1991, 1995; Tzianabos *et al.*, 1992). These two entities can be separated by iso-electrofocusing from extracts obtained by hot-phenol–water extraction followed by ethanol precipitation and separation on a Sephacryl S-300 column (Pantosti *et al.*, 1991), and high-resolution NMR spectroscopy was used to analyse the two polysaccharides (Baumann *et al.*, 1992). Polysaccharide A (PSA) is zwitterionic with a positive and a negative charge. It consists of tetrasaccharide repeating units of three sugars. Polysaccharide B (PSB) possesses one positive and two negative charges and has a hexasaccharide repeating unit. See Fig. 5 for structures of PSA and PSB.

Although it was originally reported that monoclonal antibodies specific for either polysaccharide had been produced (Pantosti *et al.*, 1991; Tzianabos *et al.*, 1992), it was subsequently reported that antibodies thought to be specific for PSB cross-reacted with PSA (Comstock *et al.*, 1999). The immunochemistry of these entities and whether they are co-expressed on the same bacterial cell is therefore unknown. The evidence for the involvement of these polysaccharides in abscess formation is discussed below.

Many strains of *B. fragilis* are refractory to the introduction of foreign DNA, which presents difficulties for genetic studies that involve the use of *E. coli*-based vectors. A spontaneous rifampicin-resistant mutant, 638R, seems to lack the restriction enzymes of most *B. fragilis* strains, since it can be conjugated with *E. coli* (Privitera *et al.*, 1979). It also lacks a visible capsule when negatively stained for light microscopy (unpublished). Mating of this strain with an *E. coli* strain that contains the *B. fragilis* transposon Tn4351 located on the suicide vector pNJR6 resulted in the production of transposon mutants that did not react with monoclonal antibody 4D5, which is specific for the PSA of *B. fragilis* NCTC 2429 (Pantosti *et al.*, 1995) and cross-reacts with 638R but not with NCTC 9343. Sequencing of the DNA at the junction of the transposon insertion revealed an open reading frame (ORF) with approximately 80% similarity to the *rmlA* genes of *Shigella flexneri* and *Salmonella enterica*. In these bacteria this gene encodes a glucose-1-phosphate thymidylate-transferase enzyme known to be involved in polysaccharide biosynthesis. A 638R *rmlA* probe identified a similar gene on the chromosome of NCTC 9343. Sequencing of the region downstream resulted in the identification of a 15 379-bp locus with 16 ORFs, designated *wcf*. Seven ORFs were identified, which probably encode sugar transferase enzymes and two *O*-acetyltransferases. A further two ORFs were thought to be involved in polysaccharide transport and polymerisation. Interestingly, a deletion mutant from which the last 10 of the 16 ORFs had been removed appeared to lack PSB but not PSA, in spite of the specificity of the 4D5 monoclonal antibody used to locate the biosynthesis region. When compared with the wild type, there was no apparent difference in the ability of the mutants to induce abscesses in a rat model of intra-abdominal infection (Comstock *et al.*, 1999).

Lipopolysaccharide and Common Antigen

The LPS of *B. fragilis* differs in composition from that of enteric bacteria in a number of respects (Lindberg *et al.*, 1990). Enterobacterial lipid A diglucosamine is bisphosphorylated, whereas in *B. fragilis* it is monophosphorylated, since the distal glucosamine residue of the lipid A molecule lacks a phosphate group. *E. coli*

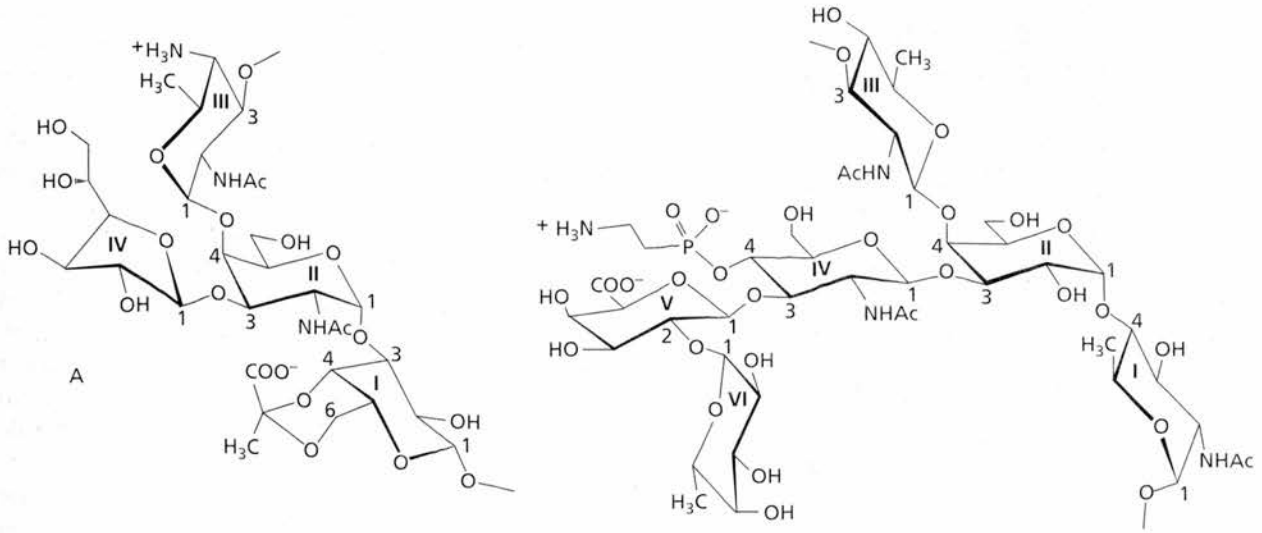


Fig. 5 Structure of PSA and PSB (see Baumann *et al.*, 1992).

has six fatty acid chains, or acyl groups, per diglucosamine backbone, which may have a chain length of 12–14 carbon atoms, whereas *B. fragilis* has 4–5 fatty acids of chain lengths 15–17 carbon atoms, with branched 3-hydroxylated and non-hydroxylated fatty acids. One of the predominant fatty acids in enterobacterial lipid A, 3-hydroxytetradecanoic acid, is thought to be lacking in *B. fragilis*. The differences in structure of the *Bacteroides* and the enterobacterial lipid A almost certainly relate to the different immunomodulatory properties of these molecules (Patrick and Larkin, 1995). The potential role of *Bacteroides* LPS in systemic inflammatory response syndrome is discussed later.

Reports are conflicting about the presence or absence of *Bacteroides* LPS of L-glycero-D-manno-heptose and keto-deoxyoctonate (KDO), both of which are present in enterobacterial LPS. KDO is almost certainly present in *Bacteroides*, but in a phosphorylated form that renders it undetectable in the standard thiobarbituric acid assay (Beckmann *et al.*, 1989; Fujiwara *et al.*, 1990). In addition, rhamnose, galactose and glucose have been reported in the core region (Lindberg *et al.*, 1990). Whether the LPS of *Bacteroides* spp. possesses an O antigen similar to that found in the enterobacteria is subject to controversy. The literature contains emphatic statements that extended repeating O antigen is absent from *Bacteroides* (Lindberg *et al.*, 1990; Comstock *et al.*, 1999). It is suggested that *Bacteroides* LPS is more like the lipooligosaccharide of, for example, *N. meningitidis* (Jennings *et al.*, 1999) or 'rough'-type mutants of enterobacteria. Other publications, however, clearly illustrate silver-stained LPS PAGE profiles with ladder patterns characteristic of the smooth LPS of *B. fragilis* (Poxton and Brown, 1986) and *B. vulgatus* (Delahooke *et al.*, 1995a: Fig. 6). A number of possible factors may explain the inability of some workers to isolate the repeating polysaccharide chains of smooth LPS. The most likely is variation within and between *Bacteroides* strains and possible loss of the ability to synthesise O antigen after repeated subculture *in vitro*. A monoclonal antibody, QUBF5, has been described, which labels a carbohydrate component with a PAGE profile similar to that of the apparent O antigen (see Fig 4: Lutton *et al.*, 1991), but it labels only a proportion of the bacteria in a population. Enrichment for two different antigenic types of the high-molecular-mass polysaccharides did not co-enrich for the QUBF5

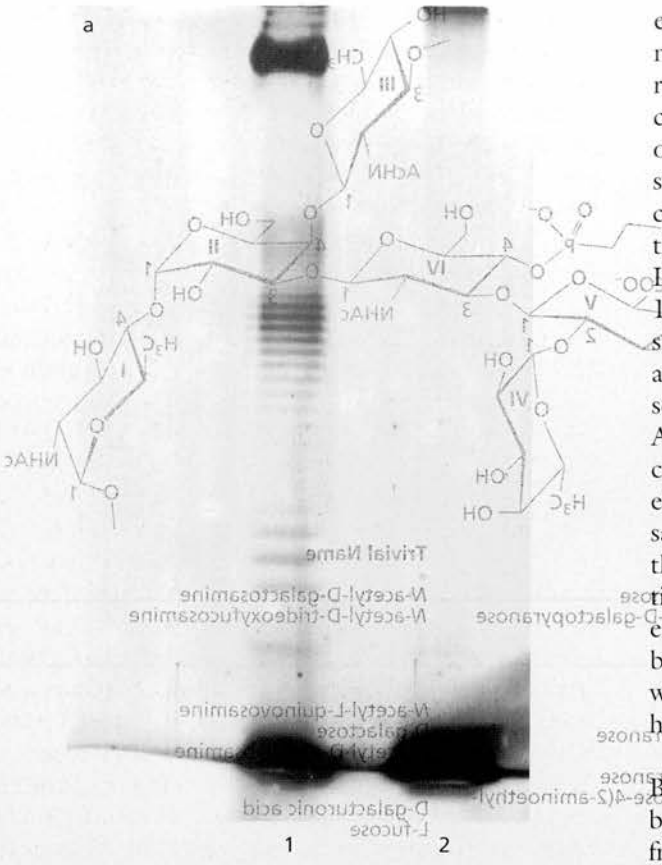


Fig. 6 Silver stained PAGE profiles: (a) *B. fragilis* strain NCTC 9344 aqueous phenol extract (track 1), phenol chloroform petroleum ether extract (track 2); (b) *B. vulgatus* strain MRPL 1985 aqueous phenol extract (track 1), phenol chloroform petroleum ether extract (track 2), triton-magnesium extract (track 3). Photographs courtesy of I.R. Poxton and D.M. Delahooke, Department of Medical Microbiology, University of Edinburgh.

epitope, as determined by immunofluorescence microscopy (Patrick *et al.*, 1999). Whether this represents within-strain antigenic variation of this component or phase variation is not known, as only one monoclonal antibody specific for this type of structure has been described. Further discussion of the conflicting data and inaccurate literature citation since the middle of the 1980s with respect to *Bacteroides* LPS (Weintraub *et al.*, 1985; Poxton and Brown, 1986) can be found in Patrick (1993). It has been suggested that some strains of *E. coli* produce not only an LPS, but also a LOS distinct from rough LPS, by a separate biosynthetic pathway (Heinrichs *et al.*, 1999). Also, the K30 capsular polysaccharide of *E. coli* may be covalently linked to core lipid A, but this is not essential either for export of this high-molecular-mass polysaccharide or for formation of a capsular structure at the cell surface (MacLachlan *et al.*, 1993). The distinction between an O antigen and a capsular or extracellular polysaccharide is therefore somewhat blurred and may finally only be resolved for *Bacteroides* when the genetic loci and pathways for biosynthesis have been identified in a range of strains.

The common antigen described by Poxton and Brown (1986) is extractable by phenol–water, runs behind the rough LPS on SDS PAGE and is distinct from the O-antigen ladder. Polyclonal mono-specific antiserum raised against immunoblot-purified material detects the antigen in *B. fragilis* strains from culture collections and recent clinical isolates from a range of geographical locations. The antiserum also labels strains of *B. ovatus* and *B. thetaiotaomicron*, by immunofluorescence microscopy, but with less intensity. This antiserum has also been used for the direct detection by immunofluorescence microscopy of *Bacteroides* in pus samples and blood culture bottles (Patrick *et al.*, 1995b). The nature of this common antigen is not known.

Fimbriae

Two types of fimbriae have been identified on *B. fragilis*, but in comparison with other bacterial species the fimbriae are not well characterised. This is probably due to poor knowledge of the precise growth conditions required for the assembly of the fimbriae. Pruzzo *et al.* (1984) described filamentous fimbriae of approximately 30 nm in diameter, and subsequently fimbriae of 4–5 nm in diameter were described (van Doorn *et al.*, 1987). Fimbria were observed by electron microscopy and immunogold labelling with fimbria-specific polyclonal antiserum. Immunoblotting after polyacrylamide gel electrophoresis revealed a fimbrial subunit of 40–42 kDa, depending on the

Fig. 7 Transmission electron micrographs of platinum gold-shadowed *B. fragilis*: (a) bacteria with a SC by light microscopy; (b) bacteria with a LC by light microscopy; (c) non-capsulate by light microscopy.

strain. Expression of the fimbrial subunit was reduced at low iron concentrations and low temperature. In a variety of strains of *B. fragilis*, grown in broth or on agar, and in a model of peritoneal infection *in vivo*, fimbrial subunits can be detected by immunoblotting. Intact fimbriae, however, have consistently been observed only by transmission electron microscopy after negative staining in agar cultures. One of the difficulties in observing fimbriae by electron microscopy relates to the interference by polysaccharide. Long strands of material are observed under the electron microscope when *B. fragilis* from broth cultures and producing a small capsule are platinum-gold shadowed or coated. Shorter strands are also observed with the LC population (Fig. 7). It remains to be determined whether these strands are purely polysaccharide or cover underlying fimbrial appendages. If the latter is the case, the polysaccharides would mask epitopes on the fimbriae, and account for erratic immunolabelling. Another possibility is that fimbrial subunits are expressed, but not always assembled into intact fimbriae (Lutton *et al.*, 1989). N-Terminal amino acid sequencing of the 40-kDa subunit of *B. fragilis* strain BE1 did not reveal any similarity with the sequences of *Porphyromonas gingivalis*, *Dichelobacter* (formerly *Bacteroides*) *nodosus* or *E. coli* type 1, K88 or CFA/1 fimbrial subunits (van Doorn *et al.*, 1992). The relationship between *B. fragilis* fimbriae and microbial attachment to host cells is considered below.

Physiology

Iron Uptake Mechanisms

The success of the majority of bacteria that grow in human hosts depends on their ability to obtain iron, which is strictly limited in availability. In the host iron is bound to transferrin and lactoferrin, and concentration of freely available iron in body fluids is about 10^{-18} mol/L (Patrick and Larkin, 1995). *B. fragilis* has not only a requirement for iron, but also an absolute requirement for haem, which is necessary for cytochrome and catalase synthesis (Morris, 1991). Although *B. fragilis* can use free haem, it is unlikely that *in vivo* there would be sufficient of this to sustain growth; it is, however, likely that *B. fragilis* is able to release haem from haemoglobin, haptoglobin–haemoglobin complexes or haemopexin–haemoglobin complexes *in vivo*. In defined medium depleted of iron by precipitation with calcium chloride, *B. fragilis* can use haemoglobin or haptoglobin–haemoglobin as a source of both iron and protoporphyrin (Otto *et al.*, 1990, 1994). This is related to the expression of a number of iron-repressible

outer-membrane proteins (OMP). Four haem-binding proteins have been isolated by haemin–agarose affinity chromatography and a further 44-kDa iron-repressible OMP is required for the haem-binding protein complex in *B. fragilis* strain BE1. *B. vulgatus*, grown under similar conditions, does not produce the same range of OMP; in particular, it lacks the 44-kDa OMP (Otto *et al.*, 1988). This may be a key factor in the predominance of *B. fragilis* over *B. vulgatus* in clinical infections (Table 2). An iron-repressible OMP of approximately 40 kDa associated with increased binding of Congo red has also been described in the type strain *B. fragilis* NCTC 9343 (Larkin *et al.*, 1988). The presence of specific antibody to the 44-kDa OMP in experimentally infected rats and also in sera from patients with *B. fragilis* infection, indicates that it is produced *in vivo* (Otto *et al.*, 1991). There was no detectable response in patients infected with other *Bacteroides* spp., such as *B. ovatus*, *B. distasonis* or *B. vulgatus*. The gene encoding the 44-kDa protein, *hupA*, from *B. fragilis* strain BE1 has been cloned and sequenced. The deduced amino acid sequence indicates that there are haem-binding motifs characteristic of haem lyase enzymes, but little similarity with other putative bacterial haem receptors (Otto *et al.*, 1996). There is no evidence that *Bacteroides* spp. produce their own iron-chelating agents such as the siderophores found in other bacteria, but the possibility cannot be ruled out. A comparison of the OMP profiles of *B. fragilis* growing *in vivo* and *in vitro* did not, however, reveal the induction of the high-molecular-mass OMP which is characteristically produced by *E. coli* grown under iron limitation (Patrick and Lutton, 1990b) and are associated with siderophore uptake.

Oxygen Sensitivity and Redox Potential

Bacteroides spp. are obligate anaerobes that, for their isolation and cultivation, require reducing conditions and, therefore, the absence of dissolved oxygen in media. To achieve this, media are usually boiled to drive off dissolved oxygen and a reducing agent, such as cysteine, is included. A redox potential of less than −42 mV at pH 7, as monitored by the redox dye resazurin, is suitable for the isolation and growth of *Bacteroides* spp. (Poxton *et al.*, 1989; Morris, 1991). Pure cultures of some strains will, however, grow in oxygen-free defined medium at an initial redox potential of +100 mV (Goldner *et al.*, 1993). Separate from this need for reducing conditions, oxygen may be directly toxic to *Bacteroides* spp., but this sensitivity is variable and some survive exposure to oxygen for quite long periods. The relative aerotolerance of some *Bacteroides* spp. probably relates to the production of superoxide dismutase and catalase. *B. fragilis* and *B. distasonis* produce catalase, but only some strains of *B. thetaiotaomicron*, *B. ovatus* and *B. eggerthi* do so, and *B. vulgatus* and *B. uniformis* are catalase negative. It has been proposed that the relative aerotolerance of *B. fragilis* contributes to its virulence, and recent clinical isolates have been reported as more aerotolerant than faecal isolates. The catalase of *B. fragilis*, KatB, is induced in late exponential phase cultures and in cultures exposed to oxidative stress when moved from anaerobic to aerobic incubation conditions. The catalase is a haemoprotein, composed of two identical subunits, each with an approximate molecular mass of 60 kDa. The catalase of *B. thetaiotaomicron* is similar but has a molecular mass of approximately 250 kDa. Cloning and sequencing of the *katB* gene of *B. fragilis* 638R has indicated amino acid sequence similarities with Gram-positive bacterial and mammalian catalases. This may reflect the taxonomic position of the *Bacteroides–Flavobacterium* phylum, but interestingly the *B. fragilis* catalase shares 71% amino acid similarity and 66% nucleotide identity with the catalase of *H. influenzae* (HktE). It has been suggested that this reflects transfer of the catalase gene into both *B. fragilis* and *H. influenzae* from a common source in the evolutionary past (Rocha and Smith, 1995). A strain with a mutation in the *katB* gene survived as well as the parent strain when exposed to oxygen, but it was more sensitive to the toxic effects of hydrogen peroxide (Rocha *et al.*, 1996). Apart from catalase, oxidative stress or exposure to hydrogen peroxide induces a further 28 proteins ranging in molecular mass from 12 to 79 kDa. If protein synthesis is inhibited by chloramphenicol, the survival of *B. fragilis* under aerobic conditions is limited (Rocha *et al.*, 1996). A number of genes are, therefore, associated with survival to oxidative stress. Some of these may also be involved in the ability of *B. fragilis* to penetrate HeLa cells (Goldner *et al.*, 1993) and in observed changes to the fermentation pathways after growth under oxidising conditions (Goldner *et al.*, 1997). Another mutant of *B. fragilis* 638R, generated by transposon mutagenesis, grows poorly in association with murine fibroblast or Chinese hamster ovary (CHO) cell line when compared with the parent strain (Tang *et al.*, 1999). This mutant was also less tolerant of exposure to oxygen than the parent strain. A 6.6 kbp fragment complemented the mutant, and sequencing of this region revealed five open reading frames which have been designated the *Bacteroides* aerotolerance operon (bat A–E). The precise function of the proteins is unknown, but they may form a membrane-associated complex involved in the generation or export of components necessary for aerotolerance.

Products

Degradative Enzymes and Outer-membrane Vesicles

The release from *Bacteroides* spp. of extracellular enzymes with the potential to degrade components of the host has been long recognised (Rudek and Haque, 1976; von Nicolai *et al.*, 1983) and, indeed, a scheme for the rapid identification of *Bacteroides* spp. based on enzymatic activities has been proposed (Hofstad, 1980). Enzymes so far described include those capable of degrading components of the host extracellular matrix, host cells and tissue such as hyaluronidase, chondroitin sulphatase, fibrinolysin, DNAase, lipases, proteases and neuraminidase. These may play a role in virulence, but in many cases their precise contribution is not proven. It may be that some of these enzymatic activities are related to the growth of *Bacteroides* spp. in the intestinal environment as part of the normal microbiota, rather than for virulence.

Proteolytic activity that may be related to either or both of virulence and survival in the intestine has been reported in *B. fragilis*. Arylamidases for the amino acids leucine, valyl-alanine and glycyl-proline are periplasmic or outer-membrane-associated in exponential phase of batch cultures. Proteolytic activity increased in the culture medium in stationary phase and was either in a soluble form or associated with particulate matter (Macfarlane *et al.*, 1992a, b). It is probable that the particle-associated enzymatic activity relates to the release of outer membrane vesicles (see below). These proteases are active against casein and gelatin (collagen type IV), but not elastin, collagen types I, II, III, VI, ovalbumin or bovine serum albumin. A protease with similar hydrolytic activity for valyl-alanine and glycyl-proline hydrolyses fibrinogen, casein and gelatin (Chen *et al.*, 1995). The purified enzyme has a molecular mass of approximately 100 kDa and the characteristics of a serine-thiol-like protease. It completely hydrolysed the α chain of fibrinogen. When the fibrinolytic activity of crude extracts of *B. fragilis*, *B. ovatus*, *B. eggerthii*, *B. uniformis* and *B. thetaiotaomicron* were compared, *B. fragilis* had the greatest activity. Since soluble fibrinogen is converted to insoluble fibrin as part of the normal blood coagulation and wound healing, this enzyme has the potential to slow clot formation, and this may play a role in virulence.

Similarly, a clear distinction between glycosidase activities of *Bacteroides* spp. that may play a role in virulence, and those that relate to degradation of intestinal components, has yet to be made. A range of glycosidases has been identified in *B. fragilis* (Table 6). For example, an endo-β-galactosidase releases oligosaccharides from the human blood group O erythrocytes (Scudder *et al.*, 1987), whereas some glycosidases degrade the carbohydrate moieties of gastric mucin. The latter may, therefore, be involved in survival within the intestine (Macfarlane and Gibson, 1991). Glycosidase, esterase, lipase (Patrick *et al.*, 1996) and neuraminidase (Domingues *et al.*, 1997) activities are associated with purified outer-membrane vesicles of *B. fragilis*. In culture, *B. fragilis* produces large amounts of single membrane-bound outer-membrane vesicles. These can be observed, by electron microscopy of ultrathin sections, and are released from the outer membrane (Fig. 1b). Immunogold labelling

Table 6 Major bacteria of the adult human faecal microbiota

Bacteria	Gram reaction	Morphology	Total viable count[a] (per g of faeces)
Bacteroides	–	Rod	10^9–10^{14}
Eubacterium	+	Rod	10^5–10^{13}
Bifidobacterium	+	Rod	10^5–10^{13}
Clostridium	+	Rod	10^3–10^{13}
Lactobacillus	+	Rod	10^4–10^{13}
Peptostreptococcus	+	Coccus	10^4–10^{13}
Ruminococcus	+	Coccus	10^5–10^{13}
Streptococcus	+	Coccus	10^7–10^{12}
Methanobrevibacter	+	Cocco–bacillus	10^7–10^{11}
Desulfovibrio	–	Rod	10^5–10^{11}
Fusobacterium	–	Rod	10^9
Enterococcus	+	Coccus	10^7
Escherichia coli	–	Rod	10^7
Prevotella/Porphyromonas	–	Rod	10^4

[a] Compiled from Willis (1991) and Macfarlane and Gibson (1994).

indicates that these vesicles carry epitopes associated with the outer membrane (Fig. 1c). Purified outer-membrane vesicles cause erythrocytes to agglutinate (Patrick *et al.*, 1996). It is, therefore, interesting to speculate that this targets, and also possibly limits, the destructive enzymatic activity to particular host cell types. It may also partly explain why *B. fragilis* does not act as a progressive rapidly acting histolytic pathogen, like other bacteria that release enzymes, such as *Clostridium perfringens*.

Polysaccharide-degrading enzymes of *B. thetaiotaomicron* have been studied in relation to their potential role in the intestinal environment. *B. thetaiotaomicron* produces two enzymes capable of degrading chondroitin sulphate and related mucopolysaccharides into disaccharides. These two enzymes have a similar molecular mass of 104–108 kDa, but they differ in their affinity for heparin. The gene for one of these chondroitin lyase enzymes has been cloned in *E. coli* (Guthrie *et al.*, 1985). Studies of starch breakdown by *B. thetaiotaomicron* indicate that the enzymes involved are not extracellular and that initial binding of the starch to an OMP complex and translocation into the periplasmic space is necessary. An operon of five genes that encode OMPs (*susC–G*) is involved in starch utilisation. At least four of the OMPs are involved in binding of starch (Reeves *et al.*, 1997).

Neuraminidases, which cleave sialic acid from oligosaccharides on host cell glycoproteins and glycolipids, may have a more subtle effect on the host and could play a key role in virulence. More than 20 naturally occurring sialic acids are known; they are formed by various substitutions and additions to neuraminic acid (Schauer, 1985). There is growing evidence that these sugar residues are involved in the biological activities of the host cells and molecules (Rademacher *et al.*, 1988). A prime example of the role of sialic acid in normal host function is its role in the movement of lymphocytes (Imai *et al.*, 1991). Microbial neuraminidases may subvert the normal function of the immune system or other systems of the host (Patrick and Larkin, 1995). The neuraminidase gene of *B. fragilis* 638R, *nanH*, has been cloned. A mutant, produced by an insertion in *nanH*, was able to revert to *nanH*$^+$, whereas another in which part of the gene had been deleted could not. The mutant capable of reversion grew as well as the parent strain in association with CHO cells and in a rat granuloma pouch model of infection, and reverted to *nanH*$^+$. Glucose limitation favoured reversion to *nanH*$^+$. The deletion mutant also grew as well as the parent strain with the CHO cell line, but grew more slowly in the rat model of infection (Godoy *et al.*, 1993). These results indicate that neuraminidase may play a role in virulence.

Enterotoxin

Enterotoxigenic *B. fragilis* were first described as a cause of acute watery diarrhoeal disease in newborn lambs (Myers *et al.*, 1984). Subsequently they were also implicated as a cause of diarrhoea in calves (Border *et al.*, 1985) and humans – particularly children (Myers *et al.*, 1987). The toxin can be detected in culture supernatants by its stimulation of secretion in ligated intestinal loops in animals and alteration of the morphology of the colonic epithelial cell line HT29/C_1 (Weikel *et al.*, 1992). The enterotoxin is a zinc-dependent metalloprotease of approximately 20 kDa. Purified toxin undergoes spontaneous breakdown by autodigestion at 37°C. The enterotoxin is active against type IV collagen (gelatin), actin, tropomyosin and fibrinogen (Moncrief *et al.*, 1995). Three isoforms of the protein have been identified. BFT-1 and BFT-2 have 92% amino acid sequence similarity. The third isoform, Korea-BFT, was identified in extra-intestinal isolates obtained in Korea and was related to BFT-2 (Chung *et al.*, 1999). The enterotoxin gene is contained in a small genetic element, termed 'pathogenicity islet', of about 6 kpb, which is bounded by 12 bp nearly perfect direct repeats. In non-toxigenic strains, a putative chromosomal integration site has been identified in a 17 bp G + C-rich region. The toxin gene encodes a 44 kDa pre-pro-toxin. It is thought that removal of a signal peptide produces a 22 kDa pro-toxin which is then cleaved to produce the 20 kDa protease. A second ORF in the pathogenicity islet that also encodes a predicted metalloprotease was identified and has a deduced amino acid sequence of 28.5% identity to the enterotoxin (Moncrief *et al.*, 1998).

In spite of the association of enterotoxin-producing strains of *B. fragilis* with diarrhoea, its importance as a cause of diarrhoea as compared with other bacterial types is a matter for debate. A study in Italy indicates that the carriage rate of enterotoxigenic *B. fragilis* in healthy adults is 15% and in adults with diarrhoea it is only 9.4%. In children it was identified in 17% of children with diarrhoea, but also in 12% of healthy children (Pantosti *et al.*, 1997). Polymerase chain reaction (PCR) assays based on the enterotoxin gene have been developed and correlate reliably with enterotoxin production in isolates (Kato *et al.*, 1996; Leszczynski *et al.*, 1997). Of 188 *B. fragilis* not associated with diarrhoea isolated in a Japanese clinical laboratory 18.6% carried the enterotoxin gene. Included in these were 64 bacteraemia isolates, of which approximately 28% carried the enterotoxin gene (Kato *et al.*, 1996). The precise role of the enterotoxin in *B. fragilis* virulence therefore remains unclear.

Bacteroides as Part of the Resident Microbiota

In normal humans *Bacteroides* spp. may colonise a number of sites, where generally their interaction with the host is considered to be either benign or potentially beneficial. Comprehensive information pertaining to aspects of the normal microbiota of humans and animals can be found in the books by Gibson (1994) and Mackie *et al.* (1997).

Although *Bacteroides* spp., including *B. fragilis* (Leszczynski *et al.*, 1997), may be isolated from the female genital tract and also transiently from skin in the anal region, the major reservoir for *Bacteroides* spp. in the human body is the large intestine (Drasar and Duerden, 1991). It is estimated that in humans there are at least 10^{11} bacteria per gram of faeces, with probably as many as 500 different bacterial types, and many of these remain to be cultured. The application of nucleic-acid-based analysis of the human microbiota, such as 16S rRNA-specific oligonucleotide probes, in combination with the detection of expression of particular components, will undoubtedly provide a comprehensive picture of the complexity of the ecology of the normal microbiota which is currently not possible with cultural techniques (Raskin *et al.*, 1997).

By far the predominant group that can be isolated from faeces are *Bacteroides* spp. of the '*B. fragilis* group', with an estimated average of 10^{11}/g of faeces. The prevalence of different bacterial types is presented in Table 6. *Bacteroides* spp. can be isolated from infants in the first day of life, but they do not begin to predominate in numbers until the introduction of solid foods (Conway, 1997). The relative incidence of different *Bacteroides* spp. in the faecal microbiota does not reflect the frequency with which these species are isolated from clinical infections, such as intra-abdominal abscesses (Table 2). *B. fragilis* is the predominant clinical isolate, whereas *B. vulgatus* may account for 40% or more of *Bacteroides* in faeces. This has led to the assumption that *B. fragilis* has determinants of virulence lacking in *B. vulgatus*, which may be the case, but in their virulence studies few workers have compared *B. vulgatus* with *B. fragilis*. Only a few studies of the mucosa-associated microbiota of the human intestine have been carried out, probably because of the difficulty in obtaining such samples, in particular from healthy individuals. Those that have been done indicate that the mucosa-adherent *Bacteroides* spp. distribution may differ considerably from that in faeces, with *B. fragilis* found in greater numbers in faeces than *B. vulgatus* (Namavar *et al.*, 1989; Poxton *et al.*, 1997).

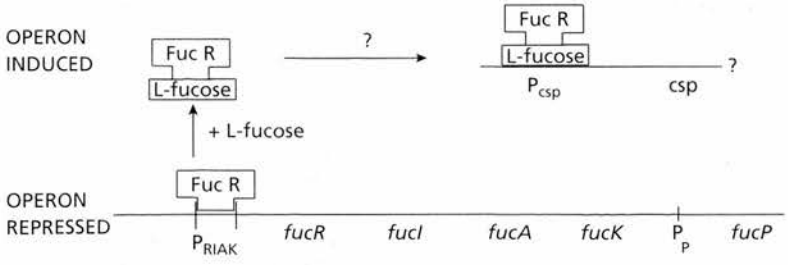
Given that there are estimated to be more bacterial cells in the normal microbiota than there are mammalian cells in the human, it is highly likely that the metabolic activity of these bacteria has a major impact on the host. It has been suggested that in terms of metabolic activity and biochemical transformations, the human large intestine rivals the liver (Macfarlane and Gibson, 1994). In the gut *Bacteroides* spp. degrade ingested material, in particular heterologous polysaccharides in plant material, that the mammalian system is incapable of degrading. This process results in the production of short-chain fatty acids, which, once absorbed, are potential substrates for energy metabolism in the intestinal mucosal epithelium. There is also evidence that bacteria in the intestine produce vitamins that can be used by the host (Drasar and Duerden, 1991). Other positive interactions of the host with the microbiota include, development of the immune system, which does not reach its full repertoire in 'germ-free' animals, and colonisation resistance to enteric pathogens provided by the resident microbiota. A detailed account of current knowledge of the complex relationship between the mucosal immune system and the intestinal microbiota is provided by Gaskins (1997).

Negative aspects of the resident microbiota include the possibility that bacterial metabolism in the intestine produces potential carcinogens (Gibson and Macfarlane, 1994). The role of anaerobic bacteria, such as *Bacteroides*, in chronic inflammatory bowel disease (IBD) of humans is controversial. There are two major types of IBD: ulcerative colitis, mainly of the colon, and Crohn's disease, which affects both the small and large intestine. A range of bacteria, including *B. vulgatus*, has been suggested as playing a role in Crohn's disease. Since the lesions in Crohn's disease are granulomatous, attention has been focused on possible mycobacterial involvement, in particular *M. paratuberculosis*. A similar situation arises with ulcerative colitis where a conclusive relationship with one type of bacterium has yet to be made. Evidence for anaerobe involvement includes the response of the condition to treatment with metronidazole (Gibson and Macfarlane, 1994). An allele of the major histocompatability complex (MHC) class I, HLA-B27, is associated with ankylosing spondylitis and reactive arthritis, and there is evidence of a relationship between patients with ankylosing spondylitis and IBD. Rats transgenic for human HLA-B27 spontaneously develop colitis, followed by arthritis and spondylitis. This does not occur, however, if the rats are raised under germ-free conditions. *B. vulgatus* was found to be a critical component of the microbiota introduced into germ-free rats for the induction of colitis (Rath *et al.*, 1996,

1999). This strongly suggests that at least one of the members of the resident microbiota could play a key role in IBD.

Although few molecular studies have been carried out concerning the fine detail of molecular interaction between the normal microbiota and humans, studies of *B. thetaiotaomicron* hint at the potential for intimate interactions and bacterial adaptation to different ecological niches along the length of the intestine. In the developing intestinal epithelium, multipotent stem cells proliferate in the crypts of Lieberkühn, and several crypts 'supply' cells for each villus. The stem cells differentiate into Paneth cells (antimicrobial-peptide-producing cells) as they migrate to the base of the crypt, and into absorptive enterocytes, enteroendocrine cells and mucus-producing goblet cells as they move up to the apex of the villus. During this differentiation, alterations occur in the composition of cell surface oligosaccharides (glycoconjugates) which can be related to the stage of differentiation of different cell types. In the mouse ileal epithelium differences in the degree of fucosylation of the glycoconjugates can be observed with the age of the germ-free mice. At 21 days, Paneth cell glycoconjugates became fucosylated in some crypts and this gradually spreads by 28 days to Paneth cells in all crypts. In contrast, when mice are raised with a conventional microbiota, not only Paneth cells, but also enterocyte and goblet-cell lineages became fucosylated. Similar development of fucosylation was noted if the germ-free mice were colonised with the normal microbiota at a later date. For example, 70-day-old germ-free mice developed fucosylation of some villi 7 days after introduction of the normal microbiota and full fucosylation 14 days later. Colonisation of the germ-free mice with a pure culture of *B. thetaiotaomicron* also restored full glycosylation, whereas an isogenic transposon insertion mutant incapable of using L-fucose as a carbon source did not. There was no difference between fucosylation of large intestinal epithelium of germ-free and colonised mice, which were both fucose positive (Bry *et al.*, 1996). Since the major site of colonisation by *Bacteroides* is the large intestine, rather than the small intestine (Drasar and Duerden, 1991; Wilson, 1997), this appears to represent specific adaptation to the small-intestinal environment. The suggestion is that the bacterium induces fucosylation of the villus glycoconjugates, then cleaves the fucose with secreted α-fucosidase, which *B. thetaiotaomicron* are known to produce, and uses the fucose as a source of carbon and energy. Further studies of *B. thetaiotaomicron* have identified FucR, a molecular sensor of L-fucose concentration, that represses the genes in the fucose utilisation operon. FucR binds fucose and it is thought that this reduces its interaction with the promoter for the L-fucose utilisation operon (Fig. 8). This is very different from the control of the fucose-utilisation gene cluster in *E. coli* which is regulated by an activator. It is not known how *B. thetaiotaomicron* causes the epithelial cells to become fucosylated, but it is postulated that there is a separate 'control of signal production' locus which is also regulated by FucR (Hooper *et al.*, 1999).

It is possible that in future the normal microbiota will no longer be seen as simple colonisers but more as an integral part of the mammalian host, inextricably linked with the normal development and functioning of the eukaryotic component. A better understanding of the normal microbiota should also allow a better understanding of the problems that arise when these



Key
P ; promoter
fucR; repressor protein
fucR; repressor gene
fucI; isomerase gene
fucA; L-fucolase aldolase gene
fucK; L-fucolase kinase gene
fucP; L-fucose permease gene
csp; control of signal production locus

Fig. 8 Fucose induction of L-fucose metabolic pathway enzymes in *Bacteroides thetaiotaomicron*.

organisms cause opportunistic infection at body sites that are not normally colonised.

Bacteroides as Pathogens

Pathogenesis by *Bacteroides* results from opportunistic infection by the normal microbiota, usually manifested by abscess formation or soft-tissue infection, which may lead to septicaemia. The possible involvement of large-scale leakage of *Bacteroides* from the intestine, in conditions such as systemic inflammatory response syndrome, has recently been examined. Enterotoxigenic *B. fragilis* associated with diarrhoea have been described (see above). The possible role of *Bacteroides* in the gastrointestinal microbiota in chronic inflammation of the bowel was also considered above. In this section, attachment mechanisms, avoidance of the host defences and immunomodulatory activities are considered. Most studies have centred on *B. fragilis*, since it is the most common clinical isolate, and the potential virulence determinants of *B. fragilis* are summarised in Table 7.

Attachment Mechanisms

B. fragilis can attach to host cells and components of the extracellular matrix. Studies of the attachment of *B. fragilis* to host cells have yielded apparently conflicting results. Polysaccharides (Riley and Mee, 1984; Vel *et al.*, 1986; Patrick *et al.*, 1996) and fimbriae (Pruzzo *et al.*, 1984) have been reported to be involved in attachment. It appears that *B. fragilis* may produce more than one type of ligand to mediate host cell attachment, and that expression of these ligands is subject to within-strain variation. *B. fragilis* populations enriched for the EDL (see above) cause haemagglutination and this is reduced by pre-treatment of the bacteria with sodium periodate, which suggests that saccharides are involved. Populations enriched for the LC do not cause haemagglutination, and it is possible to mix different proportions of these populations and correlate the degree of haemagglutination with the proportion of EDL bacteria present (Patrick *et al.*, 1988). Since recent clinical isolates vary considerably in the proportion of bacteria that express the LC, it is not surprising that there is confusion in the literature. Furthermore, the EDL population releases extracellular vesicles (see above and Fig. 1) which by themselves will cause haemagglutination (Patrick *et al.*, 1996) and also extracellular polysaccharide (Fig. 3). If quantities of vesicles or polysaccharides are present, they may block attachment sites on tissue cell lines and therefore exclude bacteria, and in assays that rely on quantification of the attached bacteria, erroneously negative results may be obtained. In assessing attachment potential it is therefore essential to have bacterial populations that are clearly defined for surface structure expression and the presence of vesicles or polysaccharide. Despite the confusion in identifying the structures involved in attachment, it is clear that

Table 7 Potential virulence determinants of *B. fragilis*

Characteristic	Bacterial component/gene[a] implicated
Attachment to host cells and extracellular matrix	Surface polysaccharides Fimbriae
Aerotolerance	Catalase (*KatB*)
Haem-binding	44-kDa OMP (*HupA*)
Tissue breakdown	
Casein hydrolysis	Protease
Gelatin hydrolysis	Protease
Fibrinogen hydrolysis	100-kDa serine-thiol-like protease
Degradation of extracellular matrix/oligosaccharides	Glycosidases, esterase lipase, neuraminidase (*nanH*)
Enterotoxicity/diarrhoea	20 kDa metalloprotease
Endotoxicity/immunomodulation	LPS
Avoidance of host defence	(Antigenically variable surface polysaccharides?)[b]
Resistance to phagocytic uptake and killing	LC (Outer membrane vesicles/extracellular polysaccharide?)
Resistance to complement-mediated killing	(Electron dense layer/extracellular polysaccharide/ outer membrane vesicles?)
Abscess formation	Extracellular polysaccharide

[a] If identified.

[b] Brackets indicate speculative involvement.

B. fragilis can attach to host cells. This may relate to both the virulence of *B. fragilis* and to its ability to colonise the intestine as part of the normal microbiota. In a comparative study of a number of isolates from faeces, abscesses and blood culture, in which the attachment ligand was not characterised, strains from all three sources caused haemagglutination and attached to human cheek epithelial cells, the intestinal cell line Intestine 407 and human polymorphonuclear leucocytes (PMNL) (Guzman *et al.*, 1997). Sialic-acid-containing glycoproteins have been implicated as potential adhesin receptors, since sialic acid and compounds that contain this sugar inhibit haemagglutination. Treatment of erythrocytes with sialidase, however, increases haemagglutination in some strains (Domingues *et al.*, 1992). This suggests that there is more than one attachment ligand in *B. fragilis* and also more than one cell receptor. There is evidence that, when grown under less reduced conditions, *B. fragilis* can penetrate tissue culture cells (Goldner *et al.*, 1993). The mechanism of penetration is unknown, but clearly this may be important for the virulence of *B. fragilis*, particularly in bacteraemia where the route of spread from the initial focus of infection to the bloodstream is unclear.

Studies of the attachment to components of the extracellular matrix are subject to the same criticism – a lack of appreciation of variability within strains, and the potential attachment of bacterial extracellular components. There is, however, clear evidence that *Bacteroides* can attach to some components of the human extracellular matrix. Binding by *B. fragilis* and *B. vulgatus* to fibronectin, collagen type I and vitronectin has been demonstrated. The binding was heat- and protease-sensitive, suggesting the involvement of a bacterial protein ligand, but fimbriae were not observed by electron microscopy (Szoke *et al.*, 1996). *B. fragilis* also binds to laminin, a major structural component of basement membranes (Eiring *et al.*, 1995).

Avoidance of the Host Defences

Resistance to complement-mediated cell lysis by *B. fragilis* in normal human serum can be observed in bacteria non-capsulate by light microscopy but with an EDL adjacent to the outer membrane (Fig. 2). The relationship between encapsulation and complement resistance is not clear, since a capsulate strain may be sensitive to complement, whereas non-capsulate populations of the same strain are resistant (Reid and Patrick, 1984; Allan and Poxton, 1994). Other *Bacteroides* spp., including *B. thetaiotaomicron*, *B. caccae*, *B. ovatus* and *B. vulgatus*, may also resist killing by serum (Bjornson, 1987; Allan and Poxton, 1994), but the proportion of bacteria that resist killing by serum can vary with the growth conditions (Allan and Poxton, 1994). Clearly, different *Bacteroides* spp. can potentially survive killing by the alternative complement pathway. Comprehensive studies of the opsonisation of *B. fragilis* with isolated components of the alternative pathway, indicate that C3 deposition occurred with C3, properdin, factors H, I, B and D. This indicates that resistance is not due to a lack of initial activation of the complement cascade, and may be due to interference with the regulation of cascade at another point in the pathway, as is the case with other bacteria that resist complement-mediated lysis (Patrick and Larkin, 1995).

Populations of *Bacteroides* enriched for the large capsule are not phagocytosed by isolated PMNL in normal human serum, but those that are non-capsulate are phagocytosed (Reid and Patrick, 1984). Though this appears to confirm the well-recognised potential antiphagocytic properties of the capsules, its relevance to infection can be questioned in the light of experiments *in vivo*. Bacteria enriched for the LC and grown *in vivo* in a chamber implanted in the mouse peritoneal cavity, not only no longer produce the LC, but on subculture they are enriched for a non-capsulate population (Patrick *et al.*, 1984). This is observable both in the presence and absence of apparently active mouse PMNL which contain phagocytosed bacteria (Patrick, 1988; Patrick *et al.*, 1995a). The small capsule-enriched population and non-capsulate bacteria do not alter capsule expression during growth *in vivo* and survive equally well in the implanted chambers. Interestingly, the influx of PMNL has no impact on the total viable count of the bacteria in the mouse peritoneal cavity model over periods of 20 days or more. Similarly, in a mouse peritoneal abscess model induced by injection of a mixture of *B. fragilis* and *E. coli* with bran, bacteria remain viable for 10 weeks or more, despite the infiltration of PMNL (Kocher *et al.*, 1996). In these abscesses, high levels of CP-10, a murine chemo-attractant which recruits neutrophils and monocytes–macrophages, and migration inhibition factor-related protein (MRP-14) which retains the recruited cells were detected. These high levels were measurable in the early acute stages of abscess formation and in chronic abscesses after 30–55 days. The release of factors such as succinic acid by *B. fragilis*, which inhibit PMNL chemotaxis (Rotstein *et al.*, 1989b), has been suggested to play a role in virulence, but this appears to be contrary to the evidence for high levels of neutrophils in models of abscess infection. Pathogenic synergy, whereby *B. fragilis* protects and enhances the survival of other facultatively anaerobic

bacteria, such as *E. coli*, has been recognised (Rotstein *et al.*, 1989a). Interestingly, abscess-derived neutrophils harbour viable bacteria and are less efficient at killing bacteria than neutrophils derived from peritoneal aspirates or peripheral blood *in vitro* (Finlay-Jones *et al.*, 1991). It is highly likely that factors other than capsules are involved in intraperitoneal survival of *B. fragilis* (Patrick *et al.*, 1995a). The potential for excreted extracellular polysaccharide and extracellular vesicles to mop up opsonins, activate complement and interact with phagocytes, to divert host defences from bacteria, should not be overlooked.

The primary opsonising molecules for attachment to PMNL have been identified as C3b and iC3b, which interact with CR3 and CR1. Purified IgM from normal humans doubles the deposition of opsonising C3 on *B. fragilis*, which suggests a potential role for IgM in alternative complement pathway activation for opsonisation of *B. fragilis* (Foreman and Bjornson, 1994). Opsonisation of *B. thetaiotaomicron* with pentameric, but not monomeric, IgM and complement components renders the capsule visible by electron microscopy after paraformaldehyde and glutaraldehyde fixation (Bjornson and Detmers, 1995). This was interpreted as evidence that the opsonins modified the capsular structure, and prevented it from being destroyed during processing for electron microscopy. The inclusion of osmium tetroxide and ruthenium red is necessary to make *Bacteroides* capsules sufficiently electron dense for observation by electron microscopy (Patrick *et al.*, 1986). Fixation with paraformaldehyde and glutaraldehyde, although necessary to retain sufficient antigenicity for immunolabelling, does not resolve the capsule even if it is present (Reid *et al.*, 1987). A more probable explanation is that the opsonising proteins render the capsule sufficiently electron dense to be visible with the electron microscope. Nevertheless, pentameric IgM enhances alternative complement pathway opsonisation of *B. thetaiotaomicron* and its subsequent adherence to PMNL.

When the extracted capsular complex of *B. fragilis* NCTC 9343, which contains PSA and PSB, is injected into the rat peritoneal cavity along with sterile caecal contents, abscesses are formed in approximately 80% of rats. The presence of both carboxyl (negative charge) and amino (positive charge) groups on the PSA is required for abscess induction in this model (Tzianabos *et al.*, 1993), and a 5-week regime of subcutaneous inoculation with the polysaccharide protects against abscess formation in at least 70% of the rats. The presence of the positive and negatively charged groups is essential for this protection. Interestingly, inoculation with *Strep. pneumoniae* capsule type 1 protects against challenge with the *B. fragilis* PS and vice versa, and indicates that this phenomenon is not restricted to *B. fragilis* (Tzianabos *et al.*, 1994). Immunity to challenge with bacteria and polysaccharide can be passively transferred by splenic lymphocytes, and T cells appear to be involved in the process (e.g. Tzianabos *et al.*, 1999). The precise role of individual T-cell types is unclear, but CD4+8+, CD4+ and CD8+ T lymphocytes may all be involved. Early reports indicated a role for CD4+8+ in abscess induction and CD8+ T cells in protection (Crabb *et al.*, 1990). More recent reports implicate CD4+ T cells in protection against abscess formation (Tzianabos *et al.*, 1999) and also conversely in an increase in abscess formation (Sawyer *et al.*, 1995). A serum IgG response to *B. fragilis* protein and polysaccharide surface antigens is detectable in a mouse chamber model of intra-peritoneal infection (Patrick *et al.*, 1995a) but it does not alter the numbers of viable bacteria in the chambers. Although the humoral immune response may not affect abscess formation, it may be an important limiting factor in bacteraemia and the systemic inflammatory response syndrome (see below). Interestingly, IgA specific for *Bacteroides* has also been detected in whole-gut lavage fluid (Poxton *et al.*, 1995). Perhaps a clearer understanding of how the *Bacteroides* spp. in the normal microbiota interact with the immune system will help to resolve the problem of the clonal immune recognition of *Bacteroides*.

Immunomodulation and Systemic Inflammatory Response Syndrome

Systemic inflammatory response Syndrome (SIRS) or 'sepsis' is the most common cause of death in patients who are critically ill in intensive care units. LPS is known to play a central role. In patients who do not have obvious septicaemia, bacteria or bacterial components appear to translocate across the ischaemic intestinal wall, and the normal intestinal microbiota are thought to be the source of the endotoxin. This sets in motion a cascade of events involving cytokines, complement and clotting factors; essentially, non-clonal immune recognition events occur. The extent of the immune response is concentration-dependent; high concentrations of bacterial immunomodulatory molecules result in what has been termed immunological 'panic'. This may ultimately result in shock, multiple organ failure and a mortality of up to 90%. It has generally been considered that the major cause of Gram-negative SIRS is the LPS of enterobacteria, but

the possible role of immunopotentiation by components of the *B. fragilis* cell envelope, such as the LPS, has not been much considered in the literature. This is largely because of the low toxicity of *B. fragilis* LPS in mouse lethality studies as compared with *E. coli* LPS. *Bacteroides* LPS is reported to be up to 10 000-fold less active than that of *E. coli* (Lindberg *et al.*, 1990). More recent investigations indicate that the extraction procedure used to prepare *Bacteroides* LPS is critical in studies of its immunomodulatory activity. Although *B. fragilis* LPS is 5000-fold less toxic in a mouse lethality model, it was 7-fold more active than phenol–water extracted *E. coli* LPS in the *Limulus* amoebocyte assay. It was also able to induce tumour necrosis factor at levels similar to *E. coli* LPS, but independently of CD14 expression. Given the high numbers of *Bacteroides* spp. in the intestinal tract, where enterobacteria may be outnumbered by up to 1000 to 1, and even allowing for the lower toxicity, it is likely that the quantitative dominance of *Bacteroides* LPS plays a role in endotoxic shock and SIRS (Delahooke *et al.*, 1995a, b). A decrease in the level of serum IgG specific for enterobacterial LPS correlates well with endotoxaemia; non-survivors of sepsis had low levels of enterobacteria-specific IgG. In a small study of 6 survivors and 6 non-survivors of sepsis, levels of IgG specific for *B. fragilis* endotoxin fluctuated more than in the healthy controls, but it is difficult to draw conclusions from such small numbers of patients; exposure to *Bacteroides* LPS had occurred in these patients (Allan *et al.*, 1995). A study of 55 patients undergoing cardiac surgery provided evidence for a decrease in anti-*B. fragilis* immunoglobulin levels during surgery, which indicates that the patients were exposed to *B. fragilis* endotoxin (Bennett-Guerrero *et al.*, 2000). In the light of these results, the potential role of *Bacteroides* in SIRS warrants further consideration.

Diagnosis

The diagnosis of *Bacteroides* and related bacteria depends largely on classical cultural methods, and in the clinical setting is usually taken no further than the genus level. For details of clinical diagnostic methods the reader is referred to the *Wadsworth Anaerobic Bacteriology Manual* (Summanen *et al.*, 1993), Brown *et al.* (1989), Wren (1991), and Jousimies-Somer *et al.* (1995). *Bacteroides* spp. can be detected in pus by direct immunofluorescence microscopy, providing the target antigen is carefully chosen and not antigenically variable (Patrick *et al.*, 1995b).

Molecular detection methods for *Bacteroides* are not as yet in general use in clinical diagnostic laboratories. PCR finger printing identification of pure culture isolates has been examined and resulted in good identification and differentiation of members of the fragilis group (Claros *et al.*, 1997). PCR-based assays for the detection of enterotoxigenic *B. fragilis* have proved reliable (e.g. Leszczynski *et al.*, 1997). Fluorescence *in situ* hybridisation (FISH) of 16S rRNA is possible with pure cultures of *B. fragilis*, but such techniques must be approached with caution when applied directly to clinical samples, as the penetration of the probe into the bacterial cell depends on the nature of encapsulating surface structures (Ramage *et al.*, 1998). Reliable direct identification of *Bacteroides* spp. and other obligate anaerobes in clinical samples is attractive, because in spite of efforts to ensure the handling and transit of samples under anaerobic conditions, a high proportion of *Bacteroides* infections may be missed because of loss of viability (Patrick *et al.*, 1995b).

Antimicrobial Agents

Antibiotics are used for the treatment of anaerobic infections and as prophylaxis for patients undergoing intestinal surgery. *Bacteroides* are intrinsically resistant to aminoglycoside antibiotics, as they are unable to transport these antibiotics. This is exploited for the selection of *Bacteroides* spp. from clinical samples that contain enterobacteria. Current understanding of the genetic systems of *Bacteroides* has come largely from studies of antibiotic resistance.

Metronidazole

Since the 1970s (e.g. Willis *et al.*, 1976) metronidazole has frequently been used for prophylaxis and for the treatment of non-clostridial anaerobic infection. Resistance to metronidazole in anaerobic bacteria is extremely low, and it has excellent tissue penetrability (Freeman *et al.*, 1997). The activity of metronidazole is restricted to anaerobes and organisms during anaerobic metabolism, as reduction of the nitro group of the drug is necessary for its antibacterial action. Reduction of the drug results in the production of radicals with the potential to accept electrons from DNA, resulting in strand breaks and helix destabilisation. In the presence of oxygen, DNA accepts electrons from the reduced drug and converts it back to the oxidised form, in a futile cycle (Edwards, 1997). False resistance to metronidazole is sometimes reported as

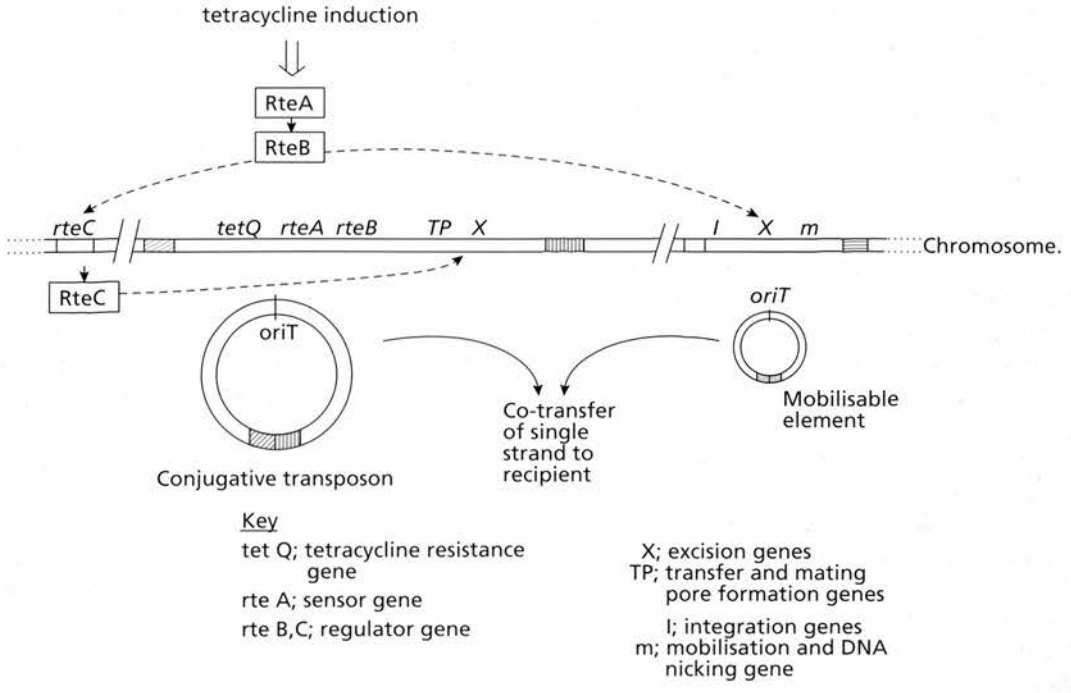


Fig. 9 Mechanism of tetracycline-inducible transfer of conjugative transposons and mobilisable elements in *Bacteroides* spp.

a result of this futile cycle if the *Bacteroides* are not cultured under strictly anaerobic conditions.

Resistance to metronidazole is rare, particularly in the USA (Dubreuil, 1996; Snydman *et al.*, 1996). It is also used for the treatment of peptic ulcer disease (Freeman *et al.*, 1997) and this wider use may lead to the development of resistance in *Bacteroides* of the normal microbiota. 5-Nitroimidazole resistance determinants (*nim* genes) have been identified on the chromosome of some strains and small plasmids (e.g. pIP417, 7.7 kbp; pIP419, 10 kpb) of *B. fragilis*. The plasmids can be transferred by a conjugation-like process in *B. fragilis*, *B. vulgatus*, *B. thetaiotaomicron* and *Prevotella* spp. (Breuil *et al.*, 1989; Sebald, 1994; Trinh *et al.*, 1996). The plasmids are not thought to be self-transmissible, but rely on conjugal helper elements, which may be chromosomal (see below and Fig. 9). Three *nim* genes that encode proteins with 85% similarity have been cloned and sequenced from plasmids and chromosomal sites (Sebald, 1994). Insertion sequences (IS) elements have been identified in the 5′ upstream region of *nim* genes, and these are involved in the regulation of expression of the antibiotic resistance gene (Smith *et al.*, 1998: Table 8). IS element involvement in the regulation of antibiotic resistance determinant expression in *Bacteroides* has also been reported for other antibiotics (see below). The mechanism of resistance to the activity of metronidazole is not fully understood.

β Lactams

The *B. fragilis* group are usually resistant to benzylpenicillin, other penicillins and many cephalosporins, with the exception of cephamycins such as cefoxitin. Carbapenems such as imipenem and meropenem are active, but some monobactams are inactive, because they lack affinity for the penicillin-binding proteins (PBP) of *B. fragilis* (Greenwood and Edwards, 1997).

Resistance to penicillin in *B. fragilis* homology group I is associated with the production of active-site serine-β lactamases distantly related to Ambler's class A. Serine-β lactamases attack the carbonyl (C = O) group of the β-lactam ring with the nucleophilic hydroxyl group of an active site serine, which results in hydrolysis of the C–N bond (Wang *et al.*, 1999). These β lactamases are susceptible to the inhibitory effects of compounds such as clavulanic acid, sulbactam and tazobactam. Cephalosporinase genes of this type – *cepA* from *B. fragilis*, *cfxA* from *B. vulgatus* and *cblA* from *B. uniformis* have been cloned and sequenced (Rogers *et al.*, 1993). In terms of amino acid sequence, these three β lactamases are more similar to each other than to all other β lactamases so far identified in other bacteria. From phylogenetic analyses, it appears that

Table 8 Examples of genetic elements of *Bacteroides* spp.

Genetic element	Size (kbp)	Resistance marker	Gene	Gene product action
Self-transmissible				
Plasmids				
pBF4	41	[a]MLS	*ermF*	RNA-methyltransferase
(associated transposon Tn4351)	(5)		[b]*tetX*	
PBI136	80	MLS	*ermFS*	
(associated transposon Tn4551)	(8.5)		[c]aadS	
Chromosomal conjugative transposons				
Tc^r DOT and related elements e.g. Tc^r	70–150	Tetracycline	*tetQ*	Ribosome protection
ERL, V479, 12256 (also termed Tn5030)		MLS	*ermF*	
Tc^r7853	70–80	Tetracycline	*tetQ*	
		MLS	*ermG*	
XBU4422	65	?	?	
Mobilisable by self-transmissible plasmid or conjugative transposons				
Plasmids				
PBFTM1O (also termed pCP1)	15			
Tn4404	5	MLS	*ermF*	
			tetX	
PBFKW1	40	Ampicillin		
Nm^r plasmids				
e.g. pIP417	7.7	5-Nitroimidazole	*nimA*	?
PIP419	10		*nimC*	?
Cryptic plasmids				
e.g. pB8–51	<8	?	?	
pBI143 (also termed pBFTM2006)				
Chromosomal integrated elements				
Transposons				
Tn4555	12	Cephalosporin,	*cfxA*	Serine protease
Tn4399	9.6	Penicillin, cefoxitin		
NBU 1, 2, 3	10–12	?	?	
Insertion sequences involved in gene activation				
IS942	1.6	β Lactam	*ccrA*	Metallo β lactamase
IS1224	>1.7	β Lactam	*cepA*	Serine protease
IS1186	1.3	β Lactam	*cfiA*	Metallo β lactamase
IS1168	1.3	5-Nitroimidazole	*nimA, B*	?
IS1169	1.3	5-Nitroimidazole	*nimD*	?
IS1170	1.6	5-Nitroimidazole	*nimC*	?
IS4351	1.2	MLS	*ermF/FS*	RNA-methyltransferase

[a] Macrolide, lincosamide, streptogramin.
[b] Silent tetracycline-resistance gene.
[c] Silent streptomycin-resistance gene.
Compiled from Sebald (1994), Salyers and Shoemaker (1996), Smith *et al.* (1998).

these *Bacteroides* enzymes evolved at an earlier stage, which is consistent with the early evolutionary divergence of the *Bacteroides*.

Determinants for these β lactamases may be chromosomal, but can be transferred between species of the *B. fragilis* 'group'. The co-transfer of ampicillin resistance with a conjugative transposon that carries a tetracycline resistance marker has been demonstrated. Similarly, the *cfxA* gene, which encodes a cephalosporinase that degrades cephalosporins, penicillins and cefoxitin, is located on a mobilisable transposon, Tn4555 (see below; Sebald, 1994). Ampicillin resistance has also been located on a large transferable plasmid, but plasmid-mediated resistance to extended β lactams such as carbapenem and cefoxitin has not been reported.

B. fragilis strains that express either high or low levels of the CepA β lactam, which does not degrade

cefoxitin or imipenem, have been identified. A 40-fold increase in expression has been linked with the insertion of IS1224 upstream of the *cepA* gene. It has been suggested that the up-regulation arises from the formation of a promoter partly encoded by the IS element (Smith *et al.*, 1998).

A minority of *B. fragilis* strains produce a zinc-requiring metallo-β-lactamase of Ambler's class B, which is associated with resistance to cephamycins and carbapenems. These enzymes are resistance to the β-lactam inhibitors (clavulanic acid, sulbactam and tazobactam) and sensitive to EDTA, which chelates zinc. The proposed mechanism for the hydrolysis of the C-N bond involves an initial interaction of the nuclophile zinc hydroxide in the active site of the enzyme. This polarises the carbonyl group of the β lactam, and a second zinc interacts with the nitrogen, resulting in bond cleavage (Wang *et al.*, 1999). The chromosomal genes *cfiA* and *ccrA* have been identified. With the *cfiA* as a probe, the gene was identified in 3% of *B. fragilis* clinical isolates, which relates to the *B. fragilis* DNA homology group II (Ruimy *et al.*, 1996). These strains were lacking in the *cepA* gene that encodes the more common β lactamase of *B. fragilis* found in DNA homology group I. Differences in the composition of the OMPs and LPS of the two groups have also been noted (Edwards and Greenwood, 1997). The *cfiA* gene-positive group also carried one or more of the three IS elements IS4351, IS1186 or IS942. These are involved in the regulation of gene expression and appear to encode promoters for the antibiotic resistance genes. A promoter region has been identified in IS1186, and IS4351 encodes three promoter-like sequences. These insertion sequences were found in only 1% of the *cfiA*-negative group (Podglajen *et al.*, 1994). It has been suggested that the low incidence of the *cfiA* gene in *B. fragilis* isolates is indicative of recent acquisition of the gene (Ruimy *et al.*, 1996). Alteration of penicillin-binding proteins or outer membrane permeability may also play a role in *Bacteroides* spp. resistance to penicillin, but this remains to be fully determined (Greenwood and Edwards, 1997).

Macrolide, Lincosamide and Streptogramin

Macrolide (e.g. erythromycin), lincosamide (e.g. clindamycin) and streptogramin (e.g. pristinamycin, virginiamycin) (MLS) resistance is transferable within and between *Bacteroides* spp. by conjugative plasmid transfer (Privitera *et al.*, 1979; Sebald, 1994). MLS resistance can also be located on self-transmissible conjugative elements which also carry tetracycline resistance genes. Plasmids that have been studied include pBF4 (41 kb), pBFTM10 or pCP1 (15 kb) and pBI136 (80 kb). Plasmids pBF4 and pBI136 are self-transmissible, whereas pBFTM10 relies on mobilisation by conjugal helper elements (see below). MLS resistance is conferred by the genes *ermF* or *ermFS* (the latter differs by a single base) present on compound transposons (TN 4351, 4551 and 4400). The *ermF* gene encodes a rRNA methylase. Expression of the *erm* genes can be up-regulated by the presence of IS elements (Table 8). These *erm* genes are similar in sequence to the *erm* genes of Gram-positive bacteria. The G + C content of these genes is 32%, which is considerably less than the rest of the *B. fragilis* (42%). Tn 4351 and 4400 also carry the *tetX* gene, which is silent in *B. fragilis*, but confers tetracycline resistance in aerobically grown *E. coli*, as resistance is mediated by an NADP-requiring oxidoreductase enzyme. Tn4551 carries a silent streptomycin-resistance gene, *aadS*. The equivalent protein sequence for the *aadS* is 30% identical to the protein produced by the aadK gene of *Bacillus subtilis*. The intrinsic resistance of *Bacteroides* spp. to aminoglycoside antibiotics, because of lack of uptake, adds weight to the hypothesis that these genes have been acquired from a Gram-positive source. The IS elements that flank these transposons are either identical (IS4351 in Tn 4351 and 4551) or almost identical (IS4400 in Tn 4400). IS4351 encodes a putative transposase and is similar in G + C content to the *B. fragilis* genome (Smith *et al.*, 1998). It seems, therefore, that although the transposon genes may be of Gram-positive origin, the flanking IS elements are not.

Tetracyclines

Resistance to tetracycline is widespread in clinical and bowel *Bacteroides* spp., and mediated by the *tetQ* gene product which has a 45% amino acid identity with the *tetM* and *tetO* products in Gram-positive bacteria. TetQ and the related TetM, O, B (P) and S of other bacteria have a molecular mass of about 72 kDa and protect the ribosome from the action of tetracyclines. They have N-terminal sequence similarities with elongation factors Tu and G. The mechanism of action of TetQ is not known, but TetM increases the rate of dissociation of tetracycline from the ribosome when GTP is present (Leng *et al.*, 1997). The *tetQ* gene is carried on a large conjugative element (70 to > 150 kb) which is capable of self-transfer and may also carry the MLS resistance *erm* gene. These elements are sometimes referred to as Tet elements, Tc^r elements or conjugative transposons (CT or CTn), but it has been proposed that CTn should be used as the convention (Salyers *et al.*, 1999). They are similar to the conjugative transposons found in Gram-positive

bacteria. They differ from standard transposons in that they are larger and are not flanked by insertion sequences, but imperfect repeats of 24 bp have been identified at the ends of the element and these may be involved in the binding of excision proteins. A model has been proposed for the mechanism of conjugation in which the CTn is excised from the chromosome and forms a non-replicating plasmid-like covalently closed circle. This is transferred in a manner similar to a plasmid, with the centre of origin located near the centre of the element. A single-stranded copy enters the recipient, the second strand is synthesised to form a double-stranded circular element which integrates into the recipient chromosome, without duplication of the target site. This process may result in the mobilisation of other plasmids which are not self-transmissible and other unlinked chromosomal regions.

Resistance to tetracycline has increased in recent years as result of the spread of the *tetQ* gene (Leng *et al.*, 1997). TetQ can be transferred by conjugative transposition not only among *Bacteroides* such as *B. fragilis*, *B. uniformis* and *B. thetaiotaomicron*, but also between *Bacteroides* spp. and bacteria from the bovine rumen, such as *Prevotella* (*Bacteroides*) *ruminicola* (Shoemaker *et al.*, 1992) and *Enterococcus faecalis* (Leng *et al.*, 1997). Given the apparent genetic mobility of the *Bacteroides* antibiotic-resistance genes, there is the potential for the development of multiple-drug-resistant *B. fragilis*.

Genetic Systems

The development of genetic systems in *B. fragilis* has been hampered by the general lack of genetic compatibility between *E. coli* and the majority of strains of *B. fragilis* (Salyers and Shoemaker, 1996; Salyers *et al.*, 1999). *E. coli* promoters do not function in *Bacteroides*, and *E. coli* plasmids do not replicate in *Bacteroides* spp. The restriction endonucleases of *B. fragilis* preclude the introduction of *E. coli* DNA by electroporation and in many strains also by conjugation. Also, there are so far no reports of *Bacteroides* plasmids that replicate in *E. coli* (Smith *et al.*, 1998). The lack of replication of *E. coli* plasmids in *Bacteroides* has, however, been used to some advantage in the construction of suicide vectors. *Bacteroides* also appear to lack GATC sites, which are methylated at the adenine residue by the Dam methylase in *E. coli*. This process is important in *E. coli* for the repair of damaged DNA, regulation of gene expression and some gene re-arrangements (Holdman *et al.*, 1976; Patrick and Larkin, 1995). It has been suggested that this may explain the instability of *Bacteroides* DNA in cosmid clones in *E. coli* hosts (Shoemaker *et al.*, 2000). The apparent general genetic incompatibility probably reflects the taxonomic distance between the two genera. *Bacteroides* spp. fall into the *Bacteroides–Flavobacterium* phylum (Woese, 1987). It is thought that this phylum, which includes the majority of Gram-negative organisms, such as the enteric bacteria, *E. coli* and the pseudomonads, diverged from other eubacteria early in evolutionary terms, well before the divergence of the Gram-positive bacteria from the Purple bacteria.

In spite of this, the study of the potential spread of antibiotic-resistance determinants has lead to the recognition of two key features in *Bacteroides*. First, they possess a number of different types of potentially mobile genetic elements. These include the unusual chromosomal transposons that are mobilisable by other genetic elements and have not yet been described in other genera (see Table 8, Fig. 8 and below). Second, there is a relationship between insertion sequences and up-regulation of antibiotic gene expression, but whether this up-regulation occurs with other genes remains to be determined. Interestingly in *D. nodosus*, the causative agent of ovine footroot, chromosomal genetic elements (*int*A, B and C elements) that up-regulate virulence determinants have been described (Whittle *et al.*, 1999).

Mobile Genetic Elements

A small number of self-transmissible plasmids that carry antibiotic resistance genes have been identified (e.g. pBF4 which carries MLS resistance), but much of the movement of genetic elements among *Bacteroides* appears to be driven by large chromosomal conjugative transposons. The most extensively studied of these are the Tc^r elements that carry the tetracycline resistance gene, *tetQ*, but other 'cryptic' elements have also been identified (e.g. XBU4422 in *B. uniformis*). The chromosomal insertion site of these elements seems to be non-random and restricted to 3–7 chromosomal sites. These elements carry the genes necessary for self-transmission by conjugation, and the mechanism of transfer is thought to involve a circular, non-replicating intermediate form (Salyers and Shoemaker, 1996). Unusually, the frequency of conjugation increases more than 100-fold at low concentrations of tetracycline (about 1 μg/mL). The ORFs of two genes downstream of *tetQ* are similar to the two-component sensor/regulator genes described in a wide range of other bacteria. These have been designated *rte* (regulation of TC^r elements) A (sensor kinase) and B (regulator) (Stevens *et al.*, 1992). A third unlinked gene, *rteC*, is also involved in

regulation. These conjugative transposons mediate the conjugative transfer of other genetic elements that are not capable of transferring by themselves. They act as conjugative helpers for plasmids and also a novel class of mobile genetic elements, chromosomal mobilisable transposons, which includes the non-replicating *Bacteroides* units (NBU). *RteB* activates the excision and integration genes of the mobilisable transposons. The conjugative transposons provide the mating pore proteins. Interestingly, many of these mobilisable elements are also mobilised by the IncP plasmids of *E. coli* (Salyers and Shoemaker, 1996; Smith *et al.*, 1998).

It has been estimated that up to 50% of *Bacteroides* faecal and clinical isolates carry cryptic plasmids for which a phenotype has yet to be detected. All the cryptic plasmids so far studied carry an origin of transfer site (*oriT*), regions essential for replication and a mobilisation gene (*mob*). Although they are not self-transmissible, these genes enable them to be mobilised in *trans* by the chromosomal conjugative transposons. Antibiotic-resistance carrying plasmids that encode 5-nitroimidazole resistance and the pBFTM10 plasmid which carries MLS resistance are thought to be mobilised by the Tc^r elements.

The mobilisable transposons are 9–12 kb in size and carry the genes necessary for their own excision from the chromosome, nicking of the circular intermediate and subsequent re-integration, but not conjugation (Murphy and Malamy, 1995; Smith *et al.*, 1998). Excision may be induced by *RteB* encoded on a conjugative transposon and is therefore also up-regulated by tetracycline concentration. After excision, the mobilisable transposon forms into a non-replicating circular element, hence the assignation of the name 'non-replicating *Bacteroides* unit' (NBU) to some of these elements. The circular element is nicked by the Mob protein at the transfer origin and a single strand of the element is transferred to the recipient through the mating pore provided by the conjugative transposon. The elements identified to date include Tn4399 (*B. fragilis*), Tn4555 which encodes the *cfxA* cefoxitin resistance gene (*B. vulgatus*), NBU1 (*B. thetaiotaomicron*), NBU1 and 2 (*B. uniformis*). The NBU1 element has been sequenced. The *intN1* gene and the region of the closed ends of the circularised transposon, *attN1*, are required for the integration of NBU1 at the target chromosomal site. The *intN1* gene encodes the integrase, which has some amino acid similarity with the λ-integrase family. The mobilisation gene, *mobN1*, has been located upstream of the origin of transfer site, *oriT*. The process of excision requires, in addition to the integrase region and *oriT*, two-thirds of the *mobN1* gene region (*prmN1*) which encodes a protein involved in excision, but which is not a primase, since the circular transposon does not replicate nor is it self-transmissible. Three additional ORFs are also necessary for the excision process (Shoemaker *et al.*, 2000). The excision process therefore appears to be complex, and this may relate to the subsequent conjugation mediated by the Tc^r elements. Unlike the Tc^r elements, NBU1 integrates at a specific site located at the 3′ end of the leucine-tRNA gene.

Studies of a range of mobilisable plasmids and transposons suggest that a common feature is the cassette of genes necessary for DNA processing, and which with adjacent *oriT* form a 'mobilisation region'. Although the number and content of genes in these mobilisation regions may vary, where they are incorporated into *Bacteroides* plasmids and transposons, it enables their co-transfer with self-transmissible plasmids and Tc^r elements. Smith and colleagues (1998) provide a comprehensive review of mobile genetic elements of *Bacteroides*.

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Multiple inverted DNA repeats of *Bacteroides fragilis* that control polysaccharide antigenic variation are similar to the hin region inverted repeats of *Salmonella typhimurium*

Sheila Patrick,[1] Julian Parkhill,[4] Lisa J. McCoy,[1] Nicola Lennard,[4] Michael J. Larkin,[2] Martin Collins,[3] Matylda Sczaniecka[5] and Garry Blakely[5]

Correspondence
Sheila Patrick
s.patrick@qub.ac.uk

[1,2,3]Microbiology and Immunobiology[1], Biology and Biochemistry[2] and Food Science[3], The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK

[4]The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK

[5]Institute of Cell and Molecular Biology, The University of Edinburgh, Darwin Building, King's Buildings, Edinburgh EH9 3JR, UK

The important opportunistic pathogen *Bacteroides fragilis* is a strictly anaerobic Gram-negative bacterium and a member of the normal resident human gastrointestinal microbiota. Our earlier studies indicated that there is considerable within-strain variation in polysaccharide expression, as detected by mAb labelling. Analysis of the genome sequence has revealed multiple invertible DNA regions, designated fragilis invertible (fin) regions, seven of which are upstream of polysaccharide biosynthesis loci and are approximately 226 bp in size. Using orientation-specific PCR primers and sequence analysis with populations enriched for one antigenic type, two of these invertible regions were assigned to heteropolymeric polysaccharides with different sizes of repeating units, as determined by PAGE pattern. The implication of these findings is that inversion of the fin regions switches biosynthesis of these polysaccharides off and on. The invertible regions are bound by inverted repeats of 30 or 32 bp with striking similarity to the *Salmonella typhimurium* H flagellar antigen inversion cross-over (*hix*) recombination sites of the invertible hin region. It has been demonstrated that a plasmid-encoded Hin invertase homologue (FinB), present in *B. fragilis* NCTC 9343, binds specifically to the invertible regions and the recombination sites have been designated as fragilis inversion cross-over (fix) sites.

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INTRODUCTION

Bacteroides fragilis is the Gram-negative strictly anaerobic bacterium most frequently isolated from clinical infection, including intra-abdominal, vaginal, pilonidal, perianal and brain abscesses. It is also the most common cause of anaerobic bacteraemia, with a potential mortality of up to 19% (Redondo *et al.*, 1995). The major source of these infections is the normal resident colonic microbiota where *Bacteroides* spp. outnumber facultatively anaerobic bacteria such as *Escherichia coli* by a factor of at least 10^2–10^3 (Drasar & Duerden, 1991).

A number of factors may contribute to the virulence of *B. fragilis*; however, extracellular polysaccharides (PSs) are considered to play a key role (Patrick, 2002). Intra-strain phase variation, defined as whether a given characteristic is present or not (Saunders, 1986), is evident with respect to encapsulating surface structures (Babb & Cummins, 1978; Patrick & Reid, 1983). A large capsule (LC) and small capsule (SC) are visible by light microscopy. By electron microscopy, an encapsulating electron-dense layer (EDL) is visible adjacent to the outer membrane on bacteria non-capsulate by light microscopy (Patrick *et al.*, 1986). Expression of the different capsular types is heritable; populations can be enriched by subculture from different interfaces of Percoll step density gradients (Patrick & Reid, 1983). In addition, antigenic variation of individual capsular types can be demonstrated using mAb labelling; variable proportions of bacterial cells within these populations label with individual mAbs (Lutton *et al.*, 1991). The LC and EDL phases have shared epitopes; however, the SC is antigenically different (Reid *et al.*, 1985; Lutton *et al.*, 1991). mAb labelling indicates that there are potentially six different high-molecular-mass polysaccharides (HMMPS)

Abbreviations: EDL, electron-dense layer; HMMPS, high-molecular-mass polysaccharides; LC, large capsule; MBP, maltose-binding protein; PS, polysaccharide; SC, small capsule.

Table 1. Oligonucleotide primers used in the study

Primer	A	B	C
PS1	ccagattattatctgataat	attatcagataataatctgg	tagaaaagacgcaccccgta
PS2	gtatataatactcttataat	attataagagtattatatac	ggaacatgatcatccttcgtg
PS3	tccaataattcattgattta	taaatcaatgaattattgga	cgctcgttcttgacgatgta
PS4	ctctatatatatttgagaat	attctcaaatatatatagag	gccctggtaagcaggtcttt
PS5	gtttatagatatttgattac	gtaatcaaatatctataaac	acggatcttacgctcaccac
PS6	tcataaatacctgtggttta	taaaccacaggtatttatga	gtgcatggggatgaaattct
PS7	agatatctatctagaaatta	taatttctagatagatatct	aggaaccaaaacacggacttt

associated with both the EDL and LC. One additional HMMPS is associated with the SC. Antigenic variation has been observed in clinical isolates from a variety of anatomical sites and different geographical locations, and also in bacteria grown in an *in vivo* model of peritoneal infection (Patrick *et al.*, 1995a, b). Immunoblotting of these antigenically variable PSs after PAGE reveals patterns characteristic of heteropolymeric PSs with repeating subunits. Ladders with two different sizes of steps have been observed (Lutton *et al.*, 1991), one with a ladder pattern suggesting

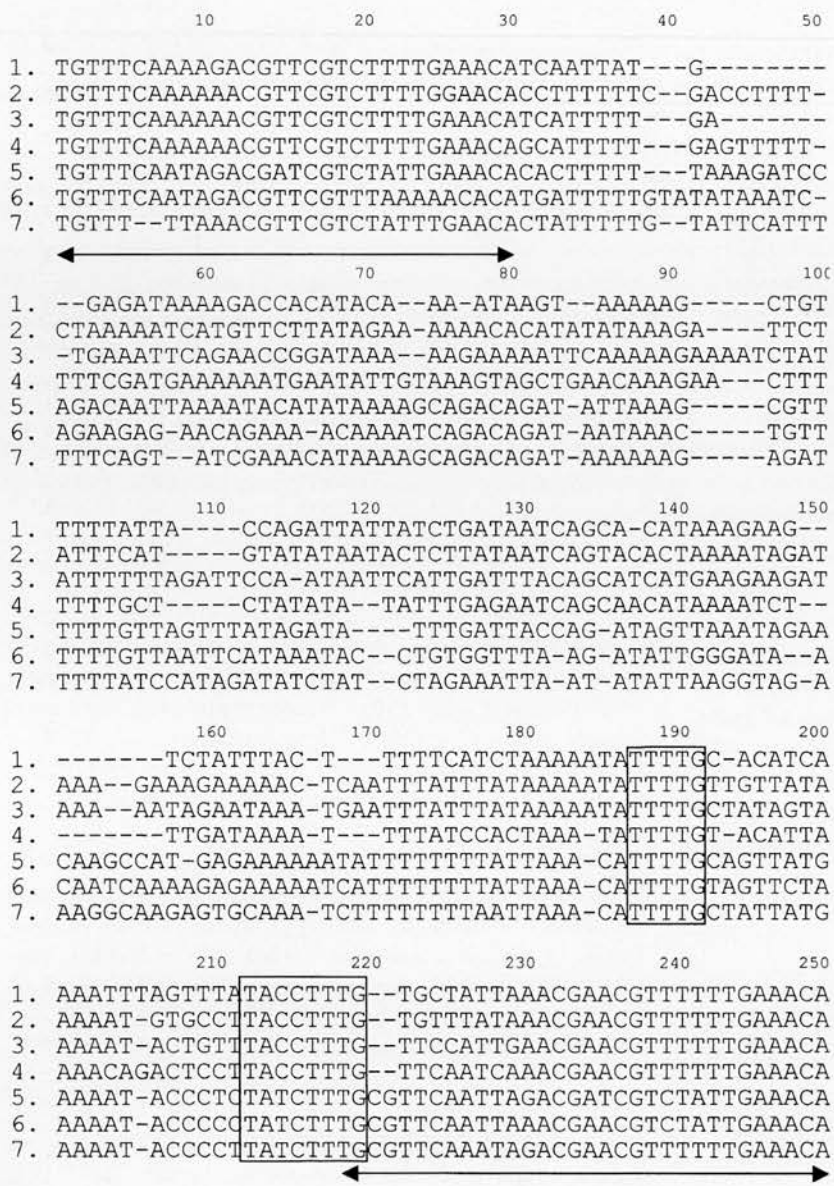

Fig. 1. Alignment of the *B. fragilis* NCTC 9343 invertible regions of DNA upstream of PS biosynthesis loci 1–7. Note inverted repeats at the right and left ends (arrows) and the *B. fragilis* consensus promoter sequence (box).

that it may be similar to the O-antigen of smooth Gram-negative bacteria identified by some workers (Poxton & Brown, 1986; Delahooke *et al.*, 1995), but not others (Lindberg *et al.*, 1990; Comstock *et al.*, 1999).

If an antigenically mixed broth culture is spread onto agar plates and the resulting colonies examined microscopically after immunofluorescence labelling, 90 % or more of the bacteria carry a given epitope in some colonies and 10 % or less in others. The proportion of bacteria expressing a given epitope within a colony is maintained on subculture of the colony into broth culture (Patrick *et al.*, 1999).

The possible mechanism underlying this complex variation was entirely unknown until the *B. fragilis* whole-genome sequencing project being carried out at the Sanger Wellcome Institute UK (http://www.sanger.ac.uk/Projects/B_fragilis/) revealed the presence of multiple regions of DNA with inverted repeat elements at either end that appear to be present in alternative orientations, within the bulk DNA supplied for sequencing. The DNA was extracted from a defined population, enriched for the EDL/non-capsulate population, but which contained different PS antigenic types. A recent paper concluded that variation of PS expression in *B. fragilis* was probably controlled by invertible regions bound by 19 bp inverted repeats upstream of the seven putative biosynthesis loci (Krinos *et al.*, 2001). We present evidence that the inverted repeat regions at these loci are in fact 30 or 32 bp in length and similar to the *Salmonella typhimurium* H flagellar antigen inversion cross-over (*hix*) recombination sites of the invertible hin region of *S. typhimurium* that controls biphasic flagellar antigenic variation (Zieg *et al.*, 1977). We also show, using mAbs, that PS epitope expression associated with defined subpopulations is related to specific *S. typhimurium* H flagellar antigen invertase (Hin)-like inversions and that a plasmid-encoded Hin-like recombinase binds to the invertible regions.

METHODS

Bacterial strains and culture methods. *B. fragilis* NCTC 9343 (National Collection of Type Cultures, Colindale Avenue, London, UK), representative of the original strain deposited in the culture collection in 1955 and originally isolated from an abdominal abscess, was used. Identity was confirmed by partial sequencing of 16S rDNA. Bacteria were grown in defined medium (DM) broth or on DM plates (van Tassell & Wilkins, 1978) in an anaerobic cabinet [MACS Anaerobic Workstation; Don Whitley (80 % N_2, 10 % CO_2, 10 % H_2)]. Percoll density gradient enrichment was used to obtain populations that either were non-capsulate by light microscopy or produced an LC detectable by light microscopy, as described previously (Patrick & Larkin, 1993). Populations enriched for individual epitopes were obtained by picking individual colonies from DM agar plates and subculturing in DM broth (Patrick *et al.*, 1999). The proportion of bacteria labelled within a population was monitored by double immunofluorescence labelling with mAbs and polyclonal antisera as detailed previously (Patrick & Larkin, 1993). Images were viewed with a Leitz Ortholux fluorescence microscope, a Nikon DMX1200 digital camera and Lucia GF software. PAGE and immunoblotting were carried out as described previously (Lutton *et al.*, 1991).

DNA sequencing and PCR amplification. Genomic sequence data were produced by the *Bacteroides fragilis* Sequencing Group at the Wellcome Trust Sanger Institute. These data are available from http://www.sanger.ac.uk/Projects/B_fragilis/. The complete sequence and annotation of the genome will be described elsewhere.

DNA was extracted from populations enriched for one capsular phase and antigenic type, as determined by microscopy, using Qiagen Genomic-tip 100G in accordance with the manufacturer's instructions. DNA concentration was measured spectrophotometrically and diluted accordingly to 1 μg ml^{-1}. Oligonucleotide primer pairs (Table 1) were synthesized by Life Technologies. The PCR mixture used was 45 μl ABgene Reddymix containing 1·5 mM $MgCl_2$, 200 μl each dNTP, 0·025 units Thermoprime Plus DNA Polymerase, 75 mM Tris/HCl (pH 8·8), 20 mM $(NH_4)_2SO_4$ and 0·01 % (v/v) Tween 20. To the PCR mix described above, 2·5 μl each primer (20 μM) and 2·5 μl template (1 μg ml^{-1}) were added. Primer pairs were either A and C or B and C (Table 1). PCR amplification was performed for 25 cycles of 94 °C for 1 min 30 s, 50 °C for 1 min 30 s and 72 °C for 1 min 30 s using an MJ Research PTC-200 Peltier Thermal Cycler. Samples were then cooled to 4 °C and retained at this temperature until collected. After amplification, 5 μl of the amplified product was electrophoresed through a 1 % (w/v) agarose gel in 1× Tris-acetate-EDTA. PCR product bands were detected by ethidium bromide staining, visualized by UV light (Transilluminator TFX-35M; Gibco-BRL) and photographed using a Kodak DC290 Zoom Digital Camera

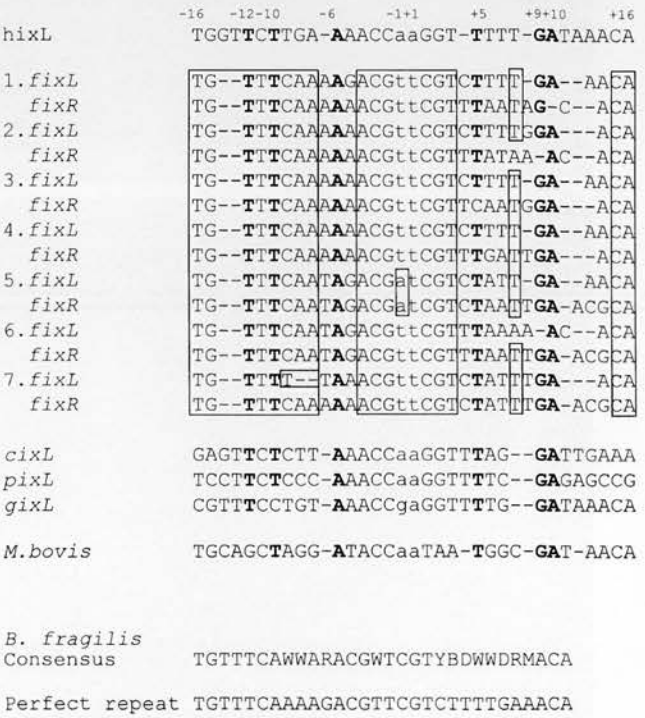

Fig. 2. Alignment of the *B. fragilis* NCTC 9343 inverted repeat regions (*fix1–7*) at the left (top strand) and right (inverse bottom strand) end of the invertible regions upstream of PS biosynthesis loci 1–7 with the promoter in the correct orientation for PS expression and top-strand left-hand inverted repeats of the 'Hin' family (*hix*, *pix*, *cix*, *gix*, *M. bovis* pilus). Central dinucleotides are in lower case. Bases that interact with the Hin invertase in *S. typhimurium* are in bold. Other conserved regions are boxed.

fitted to a Kodak EDAS290 Gel Imaging Hood. Gel images were analysed using Kodak ID Image Analysis Software Version 3.5 MAC USB. 16S rDNA primers were included as an internal standard. PCR products were purified using the MinElute PCR Purification Kit (Qiagen), according to the manufacturer's instructions. The nucleotide sequences of purified PCR products were determined using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and halfBD Terminator Sequencing Reagent (Sigma) using an ABI Prism 3100 Genetic Analyser (Applied Biosystems), according to the manufacturer's instructions.

Protein purification and gel retardation. DNA fragments corresponding to coding sequences of the *finA* and *finB* genes, generated by PCR using *Pfu* polymerase, were cloned into pMAL-c2 (NEB) to produce translational fusions with the maltose-binding protein (MBP) encoded by *malE*. *E. coli* strain DH5α was transformed with

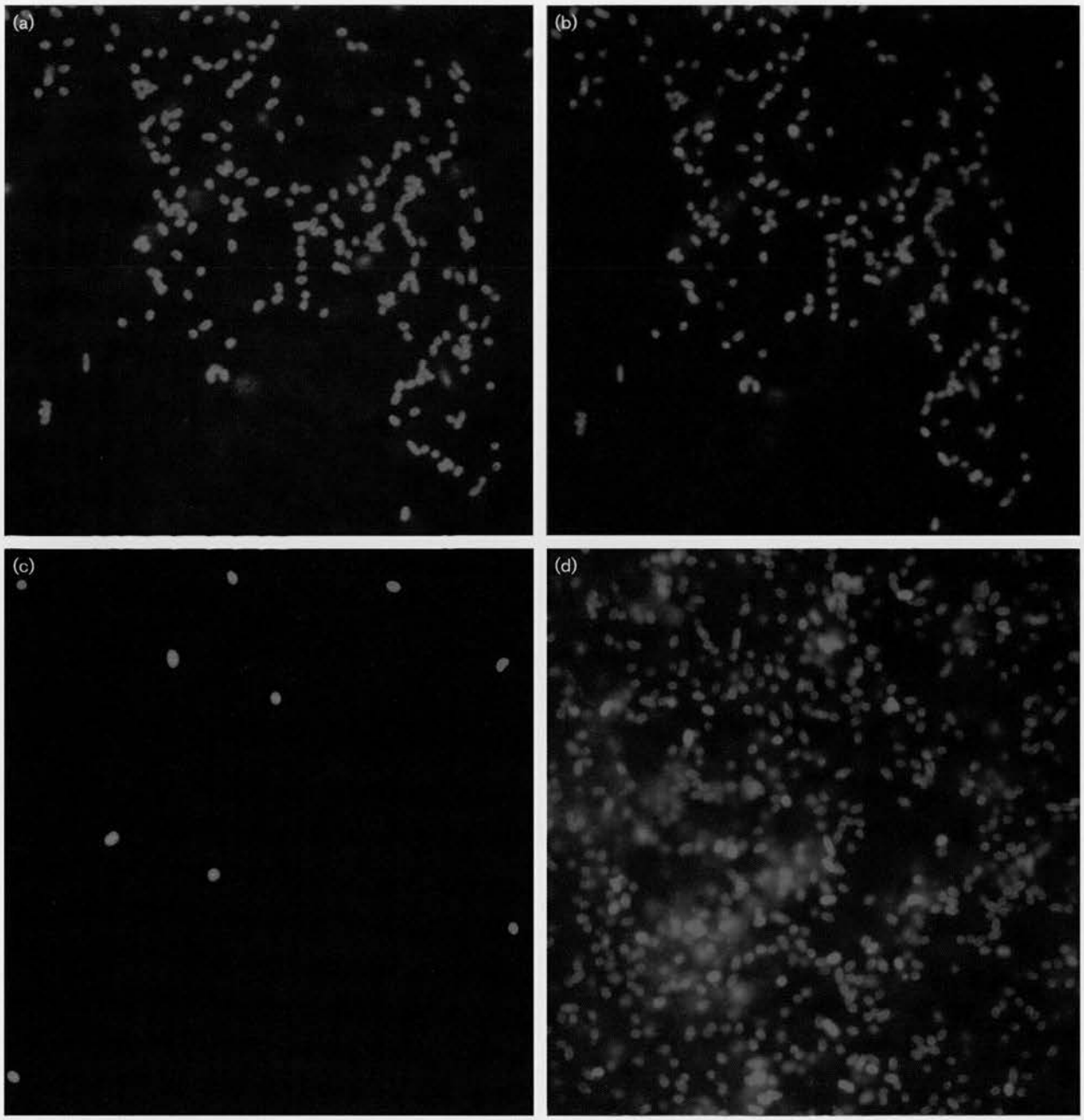

Fig. 3. Immunofluorescence micrographs of mAb QUBF5-enriched population of non-capsulate *B. fragilis* NCTC 9343 labelled with mAb QUBF5 (a), rabbit anti-*B. fragilis* polyclonal antiserum (b; same field of view as in a), mAb QUBF6 (c) and rabbit anti-*B. fragilis* polyclonal antiserum (d; same field of view as in c). The secondary antibodies are anti-mouse FITC and anti-rabbit TRITC conjugates.

plasmids that expressed the invertase-MBP fusions, grown to an OD_{600} of 0·4 and induced with 1 mM IPTG for 1 h. Cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris/HCl, pH 7·4, 200 mM NaCl, 1 mM EDTA, 1 mM PMSF), before addition of lysozyme (1 mg ml^{-1}) and sonication to produce cell lysates. After centrifugation to remove cell debris, the invertase-MBP fusion proteins were purified by affinity chromatography on amylose resin, according to the manufacturer's instructions (NEB).

Gel retardation methods were carried out as detailed by Blakely *et al.* (1993). DNA for gel retardation analysis of the entire invertible fin1 region was generated by PCR with *Pfu* polymerase, using primers that flanked the *fix1L* and *fix1R* sequences shown in Fig. 1, followed by radiolabelling using [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions (10 µl) containing approximately 0·03 pmol radiolabelled DNA were performed in 100 mM NaCl, 20 mM Tris/HCl, pH 8, 1 mM EDTA, 10 % glycerol and 100 µg poly-dIdC ml^{-1} at 37 °C for 10 min before electrophoresis through a pre-run, non-denaturing 4 % polyacrylamide gel. Binding gels were dried and then visualized by autoradiography.

RESULTS AND DISCUSSION

An alignment of the invertible regions upstream of the PS biosynthesis loci in *B. fragilis* is presented in Fig. 1. At either end of these invertible regions are AT-rich sequences containing imperfect dyad symmetry spanning 30–32 bp (inverted repeat regions). The recently proposed consensus *B. fragilis* promoter regions (Bayley *et al.*, 2000) are conserved and overlap the repeat region by 2 bp.

The inverted repeat regions, designated fragilis inversion cross-over (*fixL* and *fixR*) sites (Fig. 2) bear striking similarity to recombination sites found at the ends of some enteric bacterial invertible DNA sequences, for example, the 995 bp hin region within the chromosome of *S. typhimurium*, the invertible regions of phage Mu (Gin system), the *E. coli* K-12 e14 element (Pin system) and phage P1 (Cin system) (van de Putte & Goosen, 1992; Fig. 2). In *S. typhimurium*, the reversible expression of two antigenically different flagella, H1 and H2 (Lederberg & Iino, 1956) is controlled by inversion of the hin region which carries a promoter and is upstream of the H2 gene, and a repressor for H1. In Mu and P1 phage DNA inversion alters the tail fibres, thereby changing the phage host range (Plasterk *et al.*, 1983; Hiestand-Nauer & Iida, 1983). There is also some similarity with the *Moraxella bovis* invertible region which is within the Q/I (formerly β and α) pilus gene (Fulks *et al.*, 1990), the inversion of which mediates pilus phase and antigenic variation. There is no apparent relationship with the 9 bp repeat of the 314 bp invertible region that varies expression of type 1 fimbriae in *E. coli* which is mediated by the tyrosine family recombinases FimBE (Abraham *et al.*, 1985).

To test the hypothesis that orientation of these regions, with respect to the downstream PS biosynthesis operons, controls variation of different PSs associated with subpopulations that are definable using mAbs, a series of experiments were carried out using 'orientation-specific' PCR primers. Primer pairs (Table 1) were designed such that the PCR product would bridge the right end of the invertible region. A product from primer pair A and C would be obtained when the proposed *B. fragilis* consensus promoter (Bayley *et al.*, 2000) within the invertible region (Fig. 1) was in the correct orientation with respect to the PS biosynthesis operon ('on' position); a product from primer pair B and C would be expected when in the opposite orientation ('off' position). Populations enriched for reactivity with mAbs QUBF5, 6 and 7 by immunofluorescence microscopy were prepared; a QUBF5-enriched population is illustrated in Fig. 3. Each of these mAbs reacts with antigenically variable epitopes associated with both the LC and non-capsulate/EDL phases. After PAGE and immunoblotting, these mAbs label HMMPS with associated ladder patterns characteristic of heteropolymeric PS with repeating units (Lutton *et al.*, 1991). QUBF7 reacts with PS C (Coyne *et al.*, 2000) which lacks an invertible region upstream of the biosynthesis locus. mAb QUBF5 reacts with an HMMPS with a larger ladder step-size pattern after PAGE and immunoblotting than QUBF6 and 7 (Lutton *et al.*, 1991) (Fig. 4). Primer pairs 1A/C and 5A/C yielded a product with populations enriched for mAbs QUBF6 and 5, respectively (Fig. 5). None of the other A/C primer pairs yielded a similar product for these populations. As the enriched populations contain low

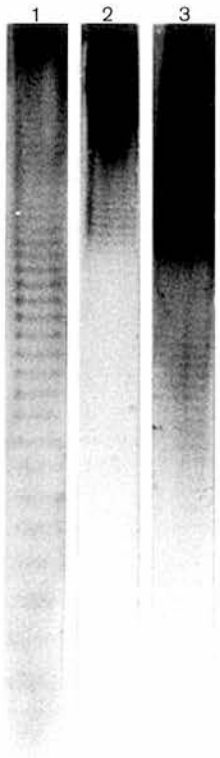

Fig. 4. Immunoblots of proteinase K extracts from *B. fragilis* NCTC 9343 after PAGE reacted with mAbs QUBF5 (lane 1), QUBF6 (lane 2) and QUBF7 (lane 3).

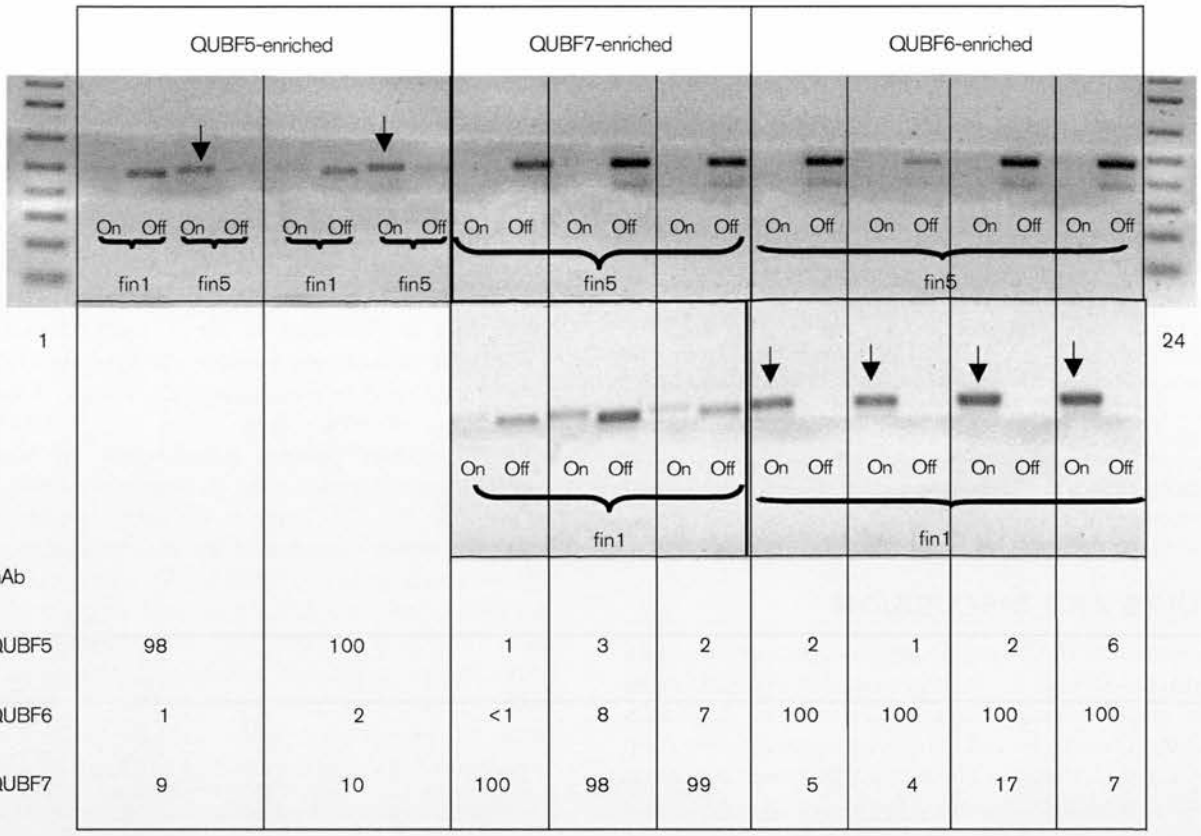

Fig. 5. PCR products, obtained from *B. fragilis* NCTC 9343 non-capsulate/EDL phase populations enriched for QUBF5, QUBF6 or QUBF7 antigenic phenotype, using primer pairs for PS biosynthesis loci 1 and 5 designed to detect fragilis invertible (fin) regions in the 'on' or 'off' position with respect to PS expression. Note products in the 'on' position for primer pairs for locus 1 and QUBF6-enriched, and locus 5 and QUBF5-enriched (arrows). Lanes 1 and 24, standards starting from the bottom at 100 bp with 100 bp increments. The numbers underneath each lane show the proportion (percentage enriched) of populations labelled with mAbs QUBF5, QUBF6 and QUBF7 as determined by immunofluorescence microscopy.

proportions of bacteria positive for other epitopes (Fig. 5), PCR product was sometimes obtained in the 'on' position from populations enriched for another epitope. For example, where 7–8 % of the population enriched for reactivity to mAb QUBF7 also reacted with mAb QUBF6, some PCR product was obtained from primer pairs 1A/C, although this was considerably less than the product from primers 1B/C (Fig. 5). The antigenic variation of the Bf5 HMMPS could explain the conflicting literature concerning the presence or absence of an 'O-antigen' in *B. fragilis* (Patrick, 2002).

Sequencing of the PCR products confirmed the sequence of the right end of the invertible region and showed that the inner half-sites of the *fixR* inverted repeats (i.e. the half-site that 'flips' during recombination within the inverting DNA) were in opposite orientations in the 'on' and 'off' positions and confirmed that site-specific recombination had occurred. This pattern of PCR product was observed for both non-capsulate/EDL and LC phases enriched for the different mAb epitopes. This suggests that the phase change associated with LC formation is not controlled by these loci. A minimum of three separate populations enriched for each phenotype was examined. Comparison of the sequence data indicates that mAbs QUBF5 and 6

```
1.fixR        TGTGCTATTAAACGaaCGTTTTTTGAAACA
PSE                    ACGAACGTTTTTTGAAACA
2.fixR        TGTGTTTATAAACGaaCGTTTTTTGAAACA
PSA                    ACGAACGTTTTTTGAAACA
3.fixR        TGTTCCATTGAACGaaCGTTTTTTGAAACA
PSH                    ACGAACGTTTTTTGAAACA
4.fixR        TGTTCAATCAAACGaaCGTTTTTTGAAACA
PSB                    ACGAACGTTTTTTGAAACA
5.fixR      TGCGTTCAATTAGACGatCGTCTATTGAAACA
PSD                TAGACGATCGTCTATTGAAACA
6.fixR      TGCGTTCAATTAAACGaaCGTCTATTGAAACA
PSF                TTAAACGAACGTCTATTGAAACACT
7.fixR      TGCGTTCAAATAGACGaaCGTTTTTTGAAACA
PSG                GTTCAAATAGACGAACGTTT
```

Fig. 6. Alignment of top-strand right-hand of *fix1–7* with 19 bp invertible regions (PS A–H) proposed by Krinos *et al.* (2001 supplementary data Table 1, available on-line). Central dinucleotides of *fixR* are in lower case.

correspond with PS E and PS D designated by Krinos *et al.* (2001) who suggested that the invertible repeat associated with the control of PS expression was 19 bp in length and of identical sequence in four of the seven regions (Krinos *et al.*, 2001 supplementary data Table 1, available on-line). The position of the 19 bp repeat relative to the suggested 30 bp repeat is illustrated in Fig. 6. There is a variable gap between the 19 bp repeat element and the consensus promoter (Bayley *et al.*, 2000) sequence situated within the invertible region, whereas the *fixL* repeat consistently overlaps the consensus promoter (Fig. 1). The asymmetry in the PS1 *fix* sites would result in the generation of different sequences if the DNA was cut in the 19 bp region rather than the 30 bp palindromic sequence. Examination of the sequences of the PCR product of PS 1 revealed a sequence consistent with the DNA inversion occurring as a result of a double-stranded DNA cut in the centre of the 30 bp (not illustrated). These sequence data were obtained with two replicate enriched populations on two separate occasions.

S. typhimurium H flagellar antigen invertase (Hin)-mediated DNA inversion occurs within a topologically defined synapse and proceeds by a concerted pair of double-stranded staggered cuts, at the central dinucleotides of each recombination site, catalysed by nucleophilic attacks mediated by the active site serine from each of the four monomers within the synapse (Heichman *et al.*, 1991). Binding specificity for Hin resides in 4 amino acid residues, invariant amongst the enteric invertases, that interact with base pairs through both the major and minor grooves of the recombination half-site (Feng *et al.*, 1994). Important base pair interactions mediated by helix 3, within the helix–turn–helix structural motif, through the major groove, include Ser174 with A10 and Arg178 with G9. Hin recognition is highly sensitive to alterations at either of these base pairs (Hughes *et al.*, 1992). These bases are highly conserved within the left half-sites of all proposed *fix* recombination sites, but are more variable in the right half-sites (Fig. 2a). Interactions of Hin with the minor groove of *hixL* involve Arg140 with A −6, Gly139 with T 5, Lys 187 with T −12 and Asn190 with T −10 and A 10. All these base pairs are conserved within the putative *fix* recombination sites. This suggested that the *fix* DNA sequences would interact with recombinases related to the enteric invertase family. Two strong candidates for invertases have been located in *B. fragilis* NCTC 9343. Fragilis invertase (Fin)A is similar to *E. coli* transposon Tn*21* resolvase TnpR (30·6 % identity in 196 aa) and FinB is homologous to *S. typhimurium* Hin (46·6 % identity in 191 aa). An alignment of FinA, FinB and Hin amino acid sequences is presented in Fig. 7. In addition to these, there are 21 proteins belonging to the 'phage integrase' family, which includes the recombinases *xerC/D* and *fimB/E*, (Kulasekara & Blomfield, 1999; Burns *et al.*, 2000), two

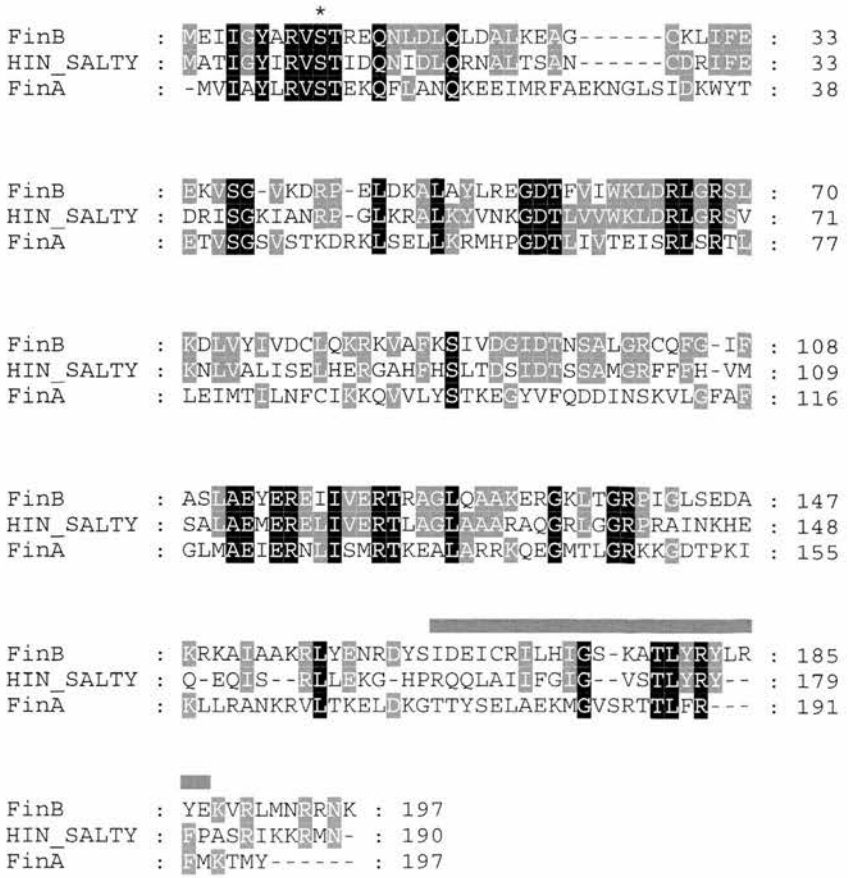

Fig. 7. Alignment of amino acid sequences of FinB, *S. typhimurium* Hin and FinA generated by CLUSTAL X. The active site residue and helix–turn–helix motif, inferred from Hin, are marked with an asterisk and a bar, respectively.

further members of the resolvase family and one protein with weak similarity to the *Moraxella* pilin inverting gene *piv*. FinB is encoded on a *B. fragilis* NCTC 9343 36·5 kbp closed circular plasmid (unpublished).

To investigate the hypothesis that the observed inversions might be mediated by a member of the enteric resolvase/invertase family, we partially purified both FinA and FinB as N-terminal fusions to the MBP. A 263 bp DNA fragment that corresponded to the invertible fin1 region was then used to assess the binding specificity of each invertase to the recombination sites. Fig. 8 demonstrates that increasing concentrations of FinB-MBP gave rise initially to a single protein/DNA complex (complex 1) that was eventually converted to a slower migrating complex (complex 2). Based on the documented attributes of the serine family of site-specific recombinases to either form dimers in solution or bind cooperatively to DNA as dimers (Spaeny-Dekking *et al.*, 1995; Blake *et al.*, 1995), one simple interpretation of the binding pattern (Fig. 8) is that a dimer of FinB-MBP was bound at either *fix1L* or *fix1R* to generate complex 1 and that at higher protein concentrations both recombination sites were occupied to give rise to complex 2. The relative amounts of the two bands, under these conditions, suggest

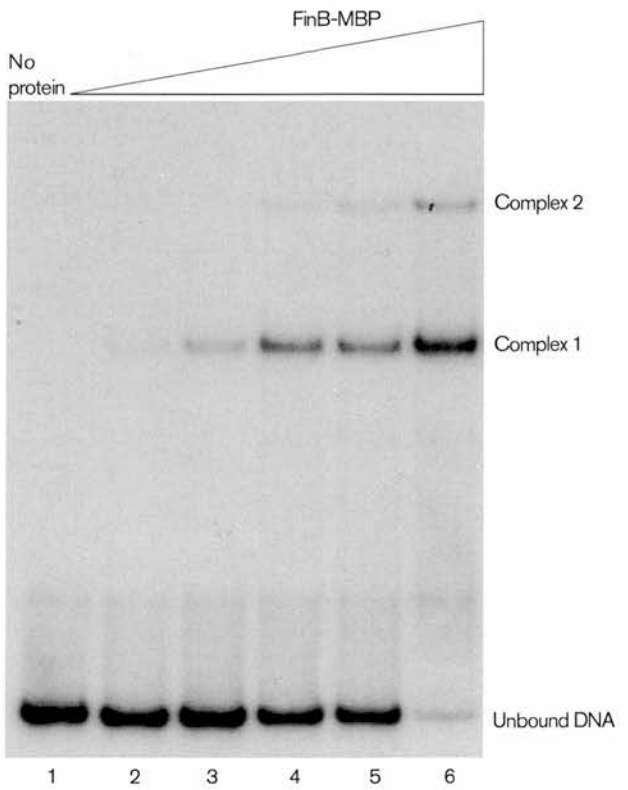

Fig. 8. Autoradiogram of *in vitro* binding between *B. fragilis* FinB-MBP and DNA, corresponding to the invertible *fix1* region, analysed by gel retardation. Radiolabelled *fix1* DNA was incubated with increasing concentrations of FinB-MBP. Lanes: 1, no FinB-MBP; 2–6, increasing concentrations of FinB-MBP.

that interactions between these complexes are not cooperative. Further analysis of the binding specificities and stoichiometry of FinB present in higher order complexes such as complex 2, however, is required to distinguish between dimer binding or formation of synaptic intermediates. FinB-MBP is also able to bind to a single recombination site derived from the *fix5* locus, while the MBP fusion to FinA did not show binding to either invertible *fix1* or *fix5* region (data not shown).

Together these data show that expression of a significant number of PS biosynthetic operons in *B. fragilis* is directly regulated by a DNA site-specific recombination inversion that appears to involve a newly identified member of the resolvase/invertase family. Whether the observed level of reversible variation of PS expression reflects the ability of *B. fragilis* to colonize the hostile environment of the human host as an opportunistic pathogen, as a member of the normal intestinal microbiota or both, remains to be determined. Variation of the surface PSs in *B. fragilis* undoubtedly occurs during natural infection by *B. fragilis*. Direct examination of pus drained from abdominal, perianal, ischiorectal, pilonidal, vaginal, Bartholin's, diverticular, colostomy, groin and neoplasm abscesses as well as blood culture bottles and labelled with six different PS-specific mAbs, revealed diverse labelling patterns. No single PS or group of PSs could be related to infection at one particular site (Patrick *et al.*, 1995b).

The evolutionary origin of the *B. fragilis* recombination sites remains open to speculation, although it has been suggested that the Hin system in *Salmonella* arose as a result of integration of a Mu-like phage (van de Putte & Goosen, 1992). The *Bacteroidetes* diverged early, in evolutionary terms, from other eubacteria, well before the divergence of the Gram-positive bacteria from the phylum *Proteobacteria* which contains the majority of Gram-negatives such as the enteric bacteria, *E. coli* and the pseudomonads (Woese, 1987). Given the relatively limited number of other examples of reversible variation in pathogenic bacteria arising by DNA inversion, it may be that the high number of invertible sequences in *B. fragilis* reflects this early evolutionary divergence. The presence of the invertase gene, which may be responsible for controlling variable expression of multiple chromosomal loci, on a plasmid also raises a number of interesting evolutionary questions. For example, did the plasmid introduce the invertase gene or did it acquire the gene as a mechanism to ensure maintenance?

In conclusion, the putative recombination sites involved in the unprecedented level of DNA inversion observed in *B. fragilis* show striking similarity to those of the *S. typhimurium* Hin and related systems and appear to involve a plasmid-borne Hin-like invertase, FinB, which we have demonstrated binds to the invertible regions. We therefore propose that the invertible regions in *B. fragilis* are designated fin regions and the inverted repeats *fixL* or *fixR*.

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Formation of *Propionibacterium acnes* biofilms on orthopaedic biomaterials and their susceptibility to antimicrobials

Gordon Ramage[a,1], Michael M. Tunney[b,*], Sheila Patrick[a], Sean P. Gorman[b], James R. Nixon[c]

[a] *Department of Microbiology and Immunobiology, School of Medicine, Queen's University Belfast, Grosvenor Road, Belfast BT12 6BN, UK*
[b] *Clinical and Practice Research Group, School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK*
[c] *Withers Orthopaedic Centre, Musgrave Park Hospital, Stockmans Lane, Belfast BT 9 7JB, UK*

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Abstract

Failure to treat and eradicate prosthetic hip infection with systemic antibiotic regimens is usually due to the fact that the infection is associated with biofilm formation and that bacterial cells growing within a biofilm exhibit increased resistance to antimicrobial agents. In this in vitro study, we investigated the susceptibility of prosthetic hip *Propionibacterium acnes* and *Staphylococcus* spp. isolates growing within biofilms on polymethylmethacrylate (PMMA) bone cement to a range of antibiotics. All *P. acnes* isolates in the biofilm mode of growth demonstrated considerably greater resistance to cefamandole, ciprofloxacin and vancomycin. In contrast, only four of the eight *P. acnes* isolates demonstrated an increase in resistance to gentamicin. All ten *Staphylococcus* spp. isolates in the biofilm mode of growth exhibited large increases in resistance to gentamicin and cefamandole with eight of the ten isolates also exhibiting an increase in resistance to vancomycin. However, only three of the ten *Staphylococcus* spp. isolates exhibited an increase in resistance to ciprofloxacin. Biofilms were also formed on three different titanium alloys and on PMMA bone cement using *P. acnes*, *Staphylococcus epidermidis* and *Staphylococcus aureus* strains to determine if the underlying biomaterial surface had an effect on biofilm formation and the antimicrobial susceptibility of the bacteria growing within biofilms. Although differences in the rate at which the three strains adhered to the different biomaterials were apparent, no differences in biofilm antibiotic resistance between the biomaterials were observed. In the light of these results, it is important that the efficacy of other antibiotics against *P. acnes* and *Staphylococcus* spp. prosthetic hip isolates growing within biofilms on orthopaedic biomaterials be determined to ensure optimal treatment of orthopaedic implant infection.

Keywords: *Propionibacterium acnes*; Bacterial adherence; Biofilm; Polymethylmethacrylate; Titanium alloy

1. Introduction

Although total hip replacement has become commonplace in recent years, bacterial infection remains a significant complication following this procedure. In a recent study, in which we combined mild ultrasonication to dislodge the bacteria growing within adherent biofilms on the surface of the removed prosthesis with the use of strict anaerobic techniques, we cultured bacteria from 26 of 120 (22%) retrieved prostheses [1]. Sixteen of the 26 implants (62%) were infected by the anaerobic bacterium, *Propionibacterium acnes*, either as the single infecting organism (12 implants) or in combination with a Gram-positive coccus (four implants). The results of our study demonstrated for the first time that *P. acnes*, which when previously isolated had been disregarded as a skin contaminant, is associated with chronic low-grade infection of implanted biomaterials as frequently as *Staphylococcus* spp.

Attempts to treat prosthetic hip infections using systemic antibiotics usually fail as the infecting bacteria grow predominantly within a confluent biofilm on the surface of the prosthesis, rendering them resistant to currently employed antibiotics. Although previous studies have examined the antimicrobial susceptibility

*Corresponding author. Tel.: +44-28-902-7208-7; fax: +44-28-902-4779-4.
E-mail address: m.tunney@qub.ac.uk (M.M. Tunney).
[1] Current address: Department of Biological Sciences, University of Calgary, 2500 University Drive, NW, Calgary, Alberta, T2N 1N4, UK.

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of *Staphylococcus epidermidis* [2,3] and *Staphylococcus aureus* [2,4,5] biofilms formed on orthopaedic biomaterials and determined the antimicrobial susceptibility of planktonically grown *P. acnes* strains isolated from retrieved orthopaedic implants [6], there is a lack of detail with regard to *P. acnes* biofilm formation. To date, no studies have examined either the formation of *P. acnes* biofilms on orthopaedic biomaterials or determined the antimicrobial susceptibility of *P. acnes* strains growing within biofilms.

In this study we investigated *P. acnes* and *Staphylococcus* spp. biofilm formation on polymethylmethacrylate (PMMA) bone cement and titanium alloys and determined the susceptibility of these biofilms to a range of antimicrobial agents.

2. Materials and methods

2.1. Bacterial strains

Fourteen of the 18 clinical prosthetic hip isolates used in this study (HJ1 to HJ14, Table 2) were isolated as described previously [1], with a further four *P. acnes* strains (L671, L149, L1958, CK77) which had also been isolated from prosthetic joints kindly supplied by Dr. Carl Kamme, University of Lund, Sweden.

2.2. Antimicrobial agents

The following antimicrobial agents were used: Gentamicin sulphate (Sigma Chemical Co. Dorset, UK); cefamandole naftate as Kefadol® (Dista Products Ltd, Basingstoke, UK); ciprofloxacin as Ciproxin® (Bayer plc, Newbury, UK) and vancomycin as Vancocin® (Eli Lilly and Company Ltd, Basingstoke, UK).

2.3. Biomaterials

Palacos R® PMMA bone cement (Schering-Plough, Herts, UK) was prepared in accordance with the manufacturer's instructions by mixing the powdered methylmethacrylate with the liquid monomer in a bowl using a spatula. The cement mixture was immediately placed between two glass plates covered with non-adhesive backing paper (Scotch Pak, 3M, France), which were pressed together to form a sheet of cement approximately 1 mm thick. Following hardening of the cement, 1 cm^2 sections were cut with a sterile scalpel blade and stored under dark, sterile conditions at room temperature. Three different titanium–aluminium–vanadium (Ti–6Al–4 V) alloys with variable surface roughness were provided in the form of alloy discs (3.5 cm diameter) by Johnson and Johnson Medical, Ascot, UK. These were: titanium alloy 1 (TA1), 36 grit AlO_2 blast; titanium alloy 2 (TA2), 36 grit AlO_2 and wet ceramic bead blast; titanium alloy 3 (TA3), 24 grit AlO_2 and wet ceramic bead blast. TA2 was the smoothest of the three surfaces followed by TA1 and TA3 in order of increasing roughness.

2.4. In vitro development of bacterial biofilms

For comparison of biofilm formation on the four different biomaterials, one isolate from each of three major bacterial species which have been previously shown to cause prosthetic hip infection [1] (*P. acnes* [HJ 4], *S. epidermidis* [HJ 5] and *S. aureus* [HJ 9]) was selected. An overnight culture of the *P. acnes* strain which had been grown on anaerobic horse blood agar (ABA) at 37°C in an anaerobic chamber was suspended in pre-reduced cation-supplemented Mueller–Hinton broth (CSMHB, Unipath Ltd, Basingstoke, UK) at an inoculum density of approximately 1×10^8 cfu/ml. The inoculum for *S. epidermidis* and *S. aureus* was prepared by adding an aliquot (10 ml) of an overnight culture to fresh pre-warmed CSMHB (100 ml) and incubating at 37°C in an orbital incubator for 4–6 h until the exponential growth phase had been reached. This culture was then adjusted by spectrophotometric measurement to provide a final inoculum density of approximately 1×10^8 cfu/ml. Bacterial biofilms were formed by adding 5 and 25 ml of the final inoculum for each test strain to bottles containing PMMA sections and titanium alloy discs, respectively. Samples were subsequently incubated at 37°C for 0.5, 1, 2, 3, 4, 6, 8 or 18 h either aerobically (*S. epidermidis*, *S. aureus*) or anaerobically (*P. acnes*). Following biofilm formation the biomaterial samples were removed with sterile forceps and gently washed by the addition of sterile phosphate-buffered saline (PBS) three times to remove any non-adherent bacteria. The biomaterial samples were then placed in PBS and bacteria retained on the biomaterial dislodged by mild ultrasonication (5 min) in a 150 W ultrasonic bath operating at a nominal frequency of 50 Hz followed by rapid vortex mixing (30 s). Serial ten-fold dilutions were performed and viable counts estimated following the Miles and Misra drop plate count method [7]. After overnight incubation, the number of colony forming units on each biomaterial sample were counted and expressed relative to the surface area of the biomaterial sample (cfu/cm^2). All experiments were performed in triplicate with three samples of each biomaterial at each time point and the mean values were calculated.

2.5. Bactericidal activity of antibacterials against biofilm-grown cells

To test the bactericidal activity of antibiotics against the biofilm cells grown on different biomaterial substrates, the PMMA bone cement and titanium alloy

samples were incubated with the three selected test isolates (*P. acnes* [HJ 4], *S. epidermidis* [HJ 5] and *S. aureus* [HJ 9]) used for the formation of biofilms described above at 37°C for 18 h. The samples were then removed, washed by the addition of sterile PBS three times to remove any non-adherent bacteria and transferred to a series of doubling dilutions of a given antibiotic (8–1024 μg/ml) in CSMHB and incubated at 37°C for 24 h. Antibiotic-free controls were included for each biomaterial. Following incubation, the biomaterial samples were removed, washed, sonicated and the total viable count following antibiotic treatment determined as described previously. Sessile minimum bactericidal concentrations (MBCs) were determined as the lowest concentration of antibiotic giving a 99.9% reduction in viable cells (cfu/cm^2) on a biomaterial compared to the number of cfu/cm^2 on the antibiotic-free control.

The bactericidal activity of the selected antibacterial agents against the remaining 15 prosthetic hip isolates was determined as described above, following the formation of biofilms using PMMA bone cement as the biomaterial substrate.

Planktonic MBCs of all the isolates had been determined previously following evaluation of the planktonic MICs using the broth microdilution method [6,8]. In brief, aliquots from wells in the microdilution trays were plated out and after overnight incubation the planktonic MBC was defined as the lowest antibiotic concentration that produced greater than 99.9% killing of the initial inoculum.

2.6. Statistical analysis

Statistical analysis was performed using two-way analysis of variance to determine if biomaterial surface and time had a significant effect on bacterial adherence. $P<0.05$ denotes significance (Statview, Abacus Concepts Inc., CA, USA).

3. Results

Adherence of the three selected prosthetic hip isolates to the four different biomaterials are shown in Fig. 1. Analysis of variance of the results obtained showed that both biomaterial surface and time exerted a significant effect on adherence of the isolates to the biomaterials. Significant differences were observed in the adherence of *P. acnes* to each of the biomaterials. *P. acnes* adherence to TA1 was greater than to the other three biomaterials during the initial 3 h period. However, at 4 and 6 h, adherence to PMMA was greater than adherence to the other biomaterials but at 8 and 18 h adherence of *P. acnes* was again greatest to TA1. Significant differences were also observed in the adherence of *S. epidermidis* to each of the biomaterials with *S. epidermidis* adherence to TA1 significantly greater than adherence to the other biomaterials after 18 h. In contrast to the results for *P. acnes* and *S. epidermidis*, where adherence to each of the biomaterials was significantly different, significant differences in adherence of *S. aureus* were only observed between PMMA and the titanium alloys with no differences in adherence observed between the titanium alloys.

The sessile MBCs for the three representative prosthetic hip isolates growing on the four different biomaterial surfaces are shown in Table 1. The planktonic MBCs determined previously [6,8] are also shown for comparative purposes. Differences in the biomaterial surface on which the bacteria were grown did not appear to have an effect on antibiotic resistance with all sessile MBC values for a given antibiotic and test isolate being similar to within one dilution for all four biomaterials.

The sessile MBCs of all 18 isolates grown on PMMA are shown in Table 2. The planktonic MBCs determined previously [6,8] are also shown for comparative purposes. Four of the eight *P. acnes* isolates tested showed no increase in resistance to gentamicin when in the biofilm mode of growth. The remaining four isolates showed either a two- or four-fold increase in resistance. In contrast, all of the *P. acnes* isolates in the biofilm mode of growth exhibited considerably greater resistance to cefamandole, ciprofloxacin and vancomycin. All 10 *Staphylococcus* spp. isolates tested were considerably more resistant to gentamicin and cefamandole when growing in a biofilm. Similarly, eight of the ten isolates also showed an increased resistance to vancomycin when growing in a biofilm. In contrast, seven of the ten isolates tested showed no increase in resistance to ciprofloxacin when growing in a biofilm.

4. Discussion

This study is the first to describe the formation of *P. acnes* biofilm on different orthopaedic biomaterials and to determine the susceptibility of biofilm-grown *P. acnes* to a range of antimicrobial agents. The three titanium alloys compared were of the same chemical composition, but all possessed a different surface finish and, therefore, a different surface roughness. The differences in surface finish of the alloys were based on different regions of the femoral component of a prosthetic hip, which are related to aiding tissue integration and the biocompatibility of the prosthesis. *P. acnes*, *S. epidermidis* and *S. aureus* strains that had been isolated from retrieved prosthetic hip implants formed biofilms on PMMA and on the three titanium alloys. With the exception of *S. aureus*, which adhered in similar numbers to each of the three titanium alloys, differences in the biomaterial surface did have an effect

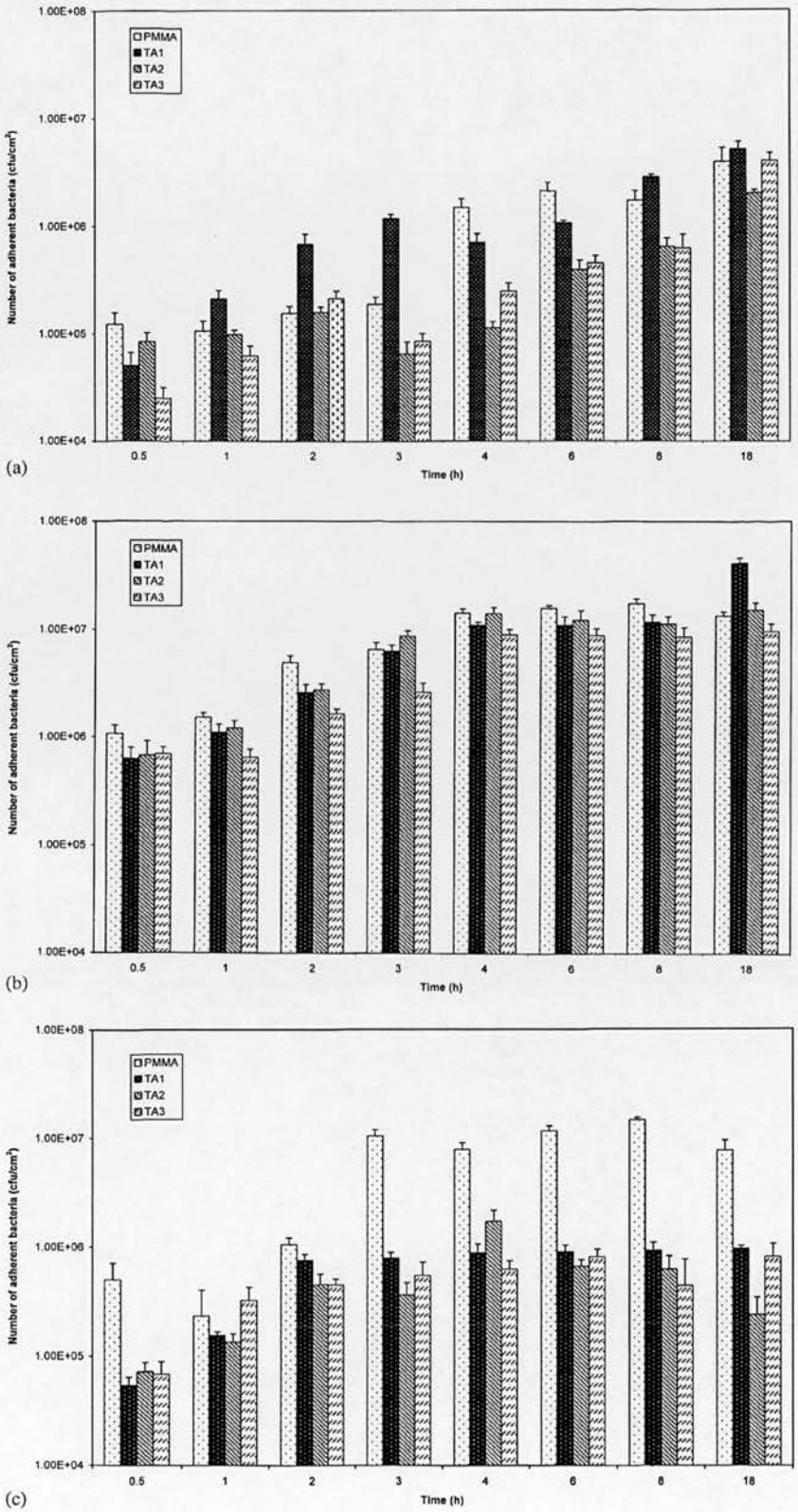

Fig. 1. Adherence of prosthetic hip isolates to biomaterials: (A) *P. acnes*; (B) *S. epidermidis*; and (C) *S. aureus*.

Table 1
Sessile minimum bactericidal concentrations of three prosthetic hip isolates grown on four different biomaterials

Strain and antibiotic	MBC[a] (μg/ml) for planktonic bacteria	MBC (μg/ml) for bacteria adherent to			
		PMMA	TA1	TA2	TA3
P. acnes (HJ 4)					
Gentamicin	16	32	16	16	32
Cefamandole	<0.5	>1024	>1024	>1024	>1024
Vancomycin	32	>1024	>1024	>1024	>1024
Ciprofloxacin	16	512	1024	1024	1024
S. epidermidis (HJ 5)					
Gentamicin	128	>1024	>1024	>1024	>1024
Cefamandole	1	1024	512	1024	512
Vancomycin	32	32	32	32	32
Ciprofloxacin	32	32	32	32	32
S. aureus (HJ 9)					
Gentamicin	32	64	64	64	64
Cefamandole	64	>1024	>1024	>1024	>1024
Vancomycin	16	>1024	>1024	>1024	>1024
Ciprofloxacin	8	256	512	512	512

[a] Minimum Bactericidal Concentration.

Table 2
Minimum bactericidal concentrations of prosthetic hip isolates grown on polymethylmethacrylate

Strain	Gentamicin		Cefamandole		Vancomycin		Ciprofloxacin	
	PMBC[a] (μg/ml)	SMBC[b] (μg/ml)	PMBC (μg/ml)	SMBC (μg/ml)	PMBC (μg/ml)	SMBC (μg/ml)	PMBC (μg/ml)	SMBC (μg/ml)
P. acnes strains								
HJ 1	32	32	1	>1024	32	>1024	8	512
HJ 2	32	32	<0.5	512	8	512	16	512
HJ 3	32	32	<0.5	>1024	16	>1024	16	256
HJ 4	16	32	<0.5	>1024	32	>1024	16	512
L671	32	128	4	>1024	8	>1024	16	512
L149	16	64	2	>1024	8	>1024	4	1024
L1958	32	64	1	256	1	>1024	8	512
CK77	32	32	1	>1024	32	>1024	4	512
S. epidermidis								
HJ 5	128	>1024	1	1024	32	32	32	32
HJ 6	1	128	1	64	16	>1024	16	16
HJ 7	32	>1024	128	>1024	32	1024	1	32
HJ 8	128	>1024	4	>1024	32	32	0.5	0.5
S. aureus (HJ 9)	32	64	64	>1024	16	>1024	8	256
S. hominis (HJ 10)	2	16	1	8	16	512	32	32
S. capitis (HJ 11)	64	256	16	64	32	64	16	16
S. haemolyticus (HJ 12)	256	512	64	>1024	1	>1024	4	4
S. sciuiri (HJ 13)	16	>1024	1	>1024	32	1024	32	64
Micrococcus sp. (HJ 14)	32	128	8	>1024	1	>1024	2	2

[a] Planktonic minimum bactericidal concentration.
[b] Sessile minimum bactericidal concentration.

on adherence of the isolates. These results are similar to the results of previous studies, which also reported differences in adherence of *S. epidermidis* and *S. aureus* to orthopaedic biomaterials [9,10]. Differences in both the adhesins expressed by the different bacteria and biomaterial surface characteristics such as surface energy, hydrophobicity, roughness and chemical composition can affect bacterial adherence to a biomaterial

and may be responsible for the differences in adherence described in this study. However, despite the fact that bacterial adherence to each of the biomaterial surfaces was significantly different, differences in biomaterial surface did not have an effect on the antimicrobial resistance of the three isolates growing within a biofilm, with few differences in sessile MBC apparent between different biomaterials. These results which indicate that once the initial biofilm has formed, the surface characteristics of the underlying biomaterial does not influence antibiotic resistance contrast with results from previous studies which reported that bacterial biofilm resistance to antibiotics was biomaterial specific [11–13].

The susceptibility of a number of additional *P. acnes* and *Staphylococcal* spp. prosthetic hip isolates growing in a biofilm on PMMA to a range of antimicrobial agents was also determined. The antibiotics studied were gentamicin, which is currently mixed with bone cement used for prosthesis fixation at the time of revision surgery, cefamandole, which is routinely used peroperatively during revision surgery and ciprofloxacin and vancomycin, which were shown to be most effective against the prosthetic hip isolates growing planktonically [6]. The high levels of gentamicin resistance determined previously [6,8] amongst planktonically grown *Staphylococcus* spp. strains may be due to the fact that gentamicin bone cement was used to fix in place the majority of implants from which the strains were isolated. Following the initial release of high levels of gentamicin from the bone cement, the subsequent low-level release of sub-inhibitory concentrations of gentamicin could have caused an increase in resistance to gentamicin amongst strains. In general, the results of this study which showed that bacteria growing within biofilms were highly resistant to the antibiotics tested are in accordance with results from previous studies examining the antimicrobial susceptibility of *S. aureus* and *S. epidermidis* biofilms [14,15]. All *Staphylococcus* spp. isolates tested exhibited large increases in resistance to gentamicin when growing in a biofilm. In contrast, biofilm-grown *P. acnes* exhibited either no or a minimal increase in resistance to gentamicin. Gentamicin resistance of sessile *Staphylococcus* spp. has also been previously observed by Chuard et al. [14] who demonstrated that sessile *S. aureus* isolates were considerably more resistant to gentamicin than the same isolates growing planktonically. Both the *P. acnes* and *Staphylococcus* spp. isolates demonstrated greatly increased resistance to cefamandole and vancomycin when growing in a biofilm. Previous studies have reported that vancomycin is unable to eradicate *S. epidermidis* growing within a biofilm [16] and suggested that this may be because as the drug enters the biofilm it is trapped in the glycocalyx and, therefore, does not reach the cell. Ciprofloxacin was the most effective antibiotic tested against *Staphylococcus* spp. prosthetic hip isolates, with seven of the ten isolates tested showing no increase in resistance to ciprofloxacin when growing in a biofilm. Earlier studies have reported that ciprofloxacin can be used effectively to eradicate bacterial biofilms [17,18]. It may, therefore, be a suitable pre-, per- and post-operative systemic antibiotic, both for prophylaxis and in the direct treatment of prosthetic joint infection caused by *Staphylococcus* spp. However, eradication of established *P. acnes* biofilms with ciprofloxacin is more problematic with all of the *P. acnes* isolates showing an increase in resistance when growing in a biofilm. At present, there is insufficient information available regarding the antimicrobial susceptibility of planktonic and biofilm-grown *P. acnes* and further studies are required in this area.

In conclusion, this in vitro study has shown for the first time that *P. acnes* is capable of forming bacterial biofilms on orthopaedic biomaterials and that the antimicrobial susceptibility of representative prosthetic hip isolates growing within a biofilm is independent of the underlying biomaterial substrate. It has also shown that no single antibiotic is effective against both *P. acnes* and *Staphylococcus* spp. isolates, both of which have been shown to be causative pathogens in prosthetic hip infection [1]. Although ciprofloxacin was effective against biofilm-grown *Staphylococcus* spp. and gentamicin effective against biofilm-grown *P. acnes*, it is important that the efficacy of other antibiotics, for example rifampicin which has been shown to have good antistaphylococcal activity and penetrative properties, be determined to ensure optimal treatment of orthopaedic implant infection.

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Propionibacterium acnes Wound Contamination at the Time of Spinal Surgery

Gregory C. McLorinan, MRCS; Josephine V. Glenn, PhD*; Michael G. McMullan, MRCS†; and Sheila Patrick, PhD**

Bacteria of the normal skin microbiota such as Propionibacterium acnes and coagulase-negative staphylococci often are dismissed as contaminants when detected in clinical samples. Propionibacterium acnes is described as a cause of spinal infection and more recently has been linked to sciatica. To date no researchers formally have examined the incidence of bacterial wound contamination during spinal surgery. Surgical specimens were removed from 79 patients having spinal surgery for analysis using agar culture detection, broth enrichment, and immunofluorescence microscopy. Bacteria were identified in 29.1% of skin samples, 21.5% of tissue samples and 16.5% of washings retrieved from operative wounds. Propionibacterium acnes was identified more frequently than Staphylococcus spp in each of the three sample types. Bacteria were detected using enrichment in 9 (11%) patients and using fluorescence microscopy in 15 (19%). The results of immunofluorescence microscopy suggest that Propionibacterium acnes detected in wounds originates from patient skin. Bacteria from contaminated wounds appeared as single cells using fluorescence microscopy; however previous work shows that bacteria from infected hip prosthesis are observed as large aggregates. Therefore, it is suggested that immunofluorescence microscopy is a useful tool to help discriminate between surgical contamination and infection.

Level of Evidence: Diagnostic study, Level I (prospective study). See the Guidelines for Authors for a complete description of levels of evidence.

From the *Department of Microbiology, School of Medicine, Queen's University, and the †Fractures Department, The Royal Victoria Hospital, Belfast, UK.
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Correspondence to: Sheila Patrick, PhD, Department of Microbiology, School of Medicine, Queen's University, Grosvenor Road, Belfast, BT12 6BN, UK. Phone: 44(0)2890 632512; Fax: 44(0)2890 635024; E-mail: s.patrick@qub.ac.uk.
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Propionibacterium acnes is an anaerobic gram-positive diphtheroid bacterium that forms part of the resident skin microbiota. Propionibacterium acnes has two distinct phenotypes, known as Types I and II.[13] The phenotypes are distinguished by serologic agglutination tests and cell wall sugar analysis. Propionibacterium acnes has become increasingly recognized as an important pathogen not only in acne,[3,11,21] but in medical-device,[2] dental,[6] ocular,[20] and neurosurgical infections,[10] and also has been linked to synovitis-acne-pustulosis-hyperostosis-osteitis (SAPHO) syndrome[14] and even sarcoidosis.[8] It has been reported previously that there is a strong association between P. acnes and failed prosthetic hip implants.[23,24]

Despite the increasing recognition of P. acnes as a pathogen, its presence in clinical samples, especially those from orthopaedic sources, might result from simple contamination. For example, sampling of clean orthopaedic wounds has shown a relatively high proportion of contamination by P. acnes,[7] whereas salvaged intraoperative blood from patients having primary hip arthroplasty has revealed contamination by P. acnes and Staphylococcus spp.[25]

Propionibacterium acnes also is associated with spinal infection. It has been isolated in cases of spinal osteomyelitis and discitis.[5,9,16] Stirling and colleagues suggested a possible link between P. acnes and sciatica.[22] Despite the apparent association between P. acnes and the spine no study to date quantifies the incidence of intraoperative wound contamination during spinal surgery.

The primary aim of this study is to ascertain the level of bacterial contamination of wound samples during spinal surgery. As well as identifying the principal bacteria involved in spinal wound contamination, possible association between the site of surgical incision and the incidence of bacterial contamination is examined. The usefulness of alternative methods to bacterial detection using agar culture, including the use enrichment broth and immunofluorescence microscopy (IFM) is assessed. Specifically we hoped to be able to demonstrate that immunofluorescence

microscopy permits the distinction between contamination and infection.

The study also describes a technique used to quantify P. acnes at different sites of the human body and also to identify the P. acnes phenotypes at those sites. The phenotypes of P. acnes identified in surgical wounds are compared to the phenotypes identified on the surface of healthy skin to help identify the possible origin of P. acnes in the surgical wound.

MATERIALS AND METHODS

Surgical material was removed from 79 consecutive patients having spinal surgery for a range of spinal conditions in Belfast between August 2002 and August 2004. Surgical material was examined for the presence of bacteria by means of agar culture, broth enrichment and immunofluorescence microscopy. A further group of 10 healthy volunteers had skin swabs taken for quantification of P. acnes at different skin sites for comparison. The study was approved by the local ethical committee.

Surgical specimens were retrieved from 79 patients: 46 (58.2%) men and 33 (41.8%) women, with indications for surgery as follows: 50 (63.3%) having discectomy for sciatica, 12 (15.2%) having laminectomy for disc degeneration, 10 (12.7%) having anterior correction of scoliosis, four (5.0%) having fixation of fracture caused by trauma, and three (3.8%) having resection of vertebral tumor. Metallic implants were inserted in all patients in the scoliosis, fracture, and tumor groups. Seven (58.3%) patients in the degeneration group had metallic implants inserted, two (16.6%) had carbon fiber implants inserted and three (25.0%) had no implant inserted. No implants were used in the sciatica group. There were 56 (70.9%) lumbar incisions, 13 (16.5%) thoracic or thoracoabdominal, six (7.6%) anterior cervical and four (5.1%) midline abdominal incisions. Sixty-eight (86.1%) were primary procedures, 10 (12.9%) were revision surgeries, and the procedure status of one (1.3%) patient was not known. Of the 10 patients who had revision surgeries seven patients had removal of recurrent disc prolapse and three had already had previous discectomy and currently were having laminectomy for spinal stenosis. Sixteen (20.3%) patients had previous spinal cannulation (epidural/intradural injection). Two (2.5%) had a previous acne history, five (6.3%) were unsure, and 72 (91.1%) had no previous acne history. Four (5.1%) patients received no prophylactic antibiotic, one (1.3%) received cefadrine, four (5.1%) received erythromycin, 29 (36.7%) received cephamandole, and 41 (51.9%) received cefuroxime. Patients were excluded from the study if infection was suspected based on clinical criteria. All surgery was done in a laminar airflow theater.

Patients' skin in all cases was prepared with Betadine (Seton Healthcare, Oldham, England) antiseptic solution. The following samples were obtained from all patients: a sample of skin (approximately 1 mm × 5 mm × 3 mm) was removed from the wound edge and a piece of tissue (approximately 3 mm × 3 mm × 3 mm) was removed from within the wound. In addition, sterile saline (10 mL) was poured into the base of the wound and was allowed to collect; then 10 mL of this fluid was aspirated for culture.

The skin sample was pulped by vortex mixing with glass beads in ¼ strength Ringer's solution containing 0.05g/l L-cysteine (QSR). Wound tissue fragments in 5 mL of QSR in universal bottles (International Scientific Supplies Limited, Bradford, UK) were exposed to ultrasonication for 5 minutes at 50 kHz in an ultrasound bath (Decon, 150W Model FS200B, Decon Laboratories Limited, Hove, UK). Skin, wound, and wash samples (0.5 mL volumes) were spread plated onto blood agar (BA; Oxoid Ltd., Basingstoke, UK) and anaerobic blood agar (ABA; Oxoid Ltd., Hampshire, England) plates in triplicate and incubated either aerobically or anaerobically for 14 days as previously described.[24] Agar culture was considered positive if bacteria were cultured on two of three aerobic or two of three anaerobic agar plates. Wound samples (0.5 mL) also were added to Robertson's cooked meat broth enrichment culture (Oxoid Ltd.), incubated for 3 weeks, plated onto BA and ABA, and these plates were examined after a further week. For the purposes of analysis, agar culture was considered the reference standard with which broth enrichment was compared. Stringent aseptic technique was adhered to at all times and any open sample processing was done within the confines of an anaerobic workstation (MACS MG 1000, Don Whitley Scientific, Shipley, UK) in which a gas mixture (80% N_2, 10% CO_2, and 10% H_2) was bubbled continuously through glutaraldehyde (2%) to prevent aerial contamination by bacteria. Pure cultures of bacteria were identified using commercially available biochemical test galleries (API, BioMèrieux, Basingstoke, UK).

A modification of the immunofluorescence microscopy (IFM) procedure described by Tunney et al was done on the wound diluents.[23] Three P. acnes antibodies were used. Antibody QUB PaIII reacts with a common antigen present on P. acnes Types 1 and 2. Antibodies QUB Pa1 and QUB Pa2 react with P. acnes Types 1 and 2 respectively. QUB PA3 cross-reacts with some Propionibacterium granulosum, Actinomyces naeslundii and A. israelii isolates. These bacteria were not, however, identified in any specimen using biochemical tests. QUB Pa1 and QUB Pa2 do not react with other propionibacterium species or a range of other related bacteria including the aerobic coryneforms.

Wound wash samples (1 mL) were centrifuged at 16,100 g for 15 minutes. The supernatant was removed, leaving a small pellet. The pellet was re-suspended in 40 μL of phosphate buffered saline (PBS; 1.061g Na_2HPO_4, 0.389g $NaH_2PO_4.2H_20$ and 8.5g NaCl per liter of H_2O). Samples (20 μL) then were applied to multiwell slides, air-dried, and fixed in 100% methanol for 10 minutes at −20°C. The fixed material was incubated for 45 minutes at 37°C with either undiluted hybridoma cell culture supernatant (30 μL) containing P. acnes reactive monoclonal antibody (QUB Pa3) or an appropriate dilution of rabbit anti-Staphylococcus spp polyclonal antiserum (30 μL). The primary antibody was gently washed off and the slide immersed in a 0.01M PBS for 15 minutes. Goat antimouse or goat antirabbit flourescein-isothiocyanate conjugate (FITC, Sigma, Poole, UK), at 1:100 dilution in PBS (30 μL) containing 0.02% (volume/volume) Evans Blue (Sigma, Poole, UK) was applied to

each well. After a further 45-minute incubation period, the slides were again washed in PBS, mounted in glycerol-PBS containing an anti-photobleaching agent (Citifluor, Agar Scientific Ltd, Stansted, UK) and examined using a Leitz Dialux 20 fluorescence microscope (Leitz, Wetzler, Germany).

When P. acnes was identified from more than one site of any patient, up to three separate colonies from each site were examined by IFM using monoclonal antibodies that react with either Type 1 (QUB Pa1) or Type 2 (QUB Pa2) P. acnes. This permitted the typing of P. acnes isolated from surgical specimens.

Skin swabs were taken from 10 healthy volunteers. Sites representing areas of common orthopaedic incision including skin over the lateral aspect of the hip, lateral aspect of the chest wall, neck, lumbar spine, and below the umbilicus were swabbed using a sterile paper template (10 cm^2). The forehead also was swabbed for comparison. The swab tip was vortex agitated in QSR (1.0 mL) and 0.5 mL was used to make a series of 10-fold dilutions in QSR. Each dilution was spread plated in triplicate onto TYG agar plates (20g/L tryptone, 10g/L yeast extract, 5 g/L glucose with 1% agar) containing 2 μg per mL furazolidone (Sigma, Poole, UK) to inhibit staphylococcal growth.[21] Plates were incubated anaerobically for 4 days at 37°C and the number of colonies was recorded. Immunoreaction was done as previously described, with minor modification.[19] In brief, nitrocelluose was blocked with 0.01 M PBS containing 0.05% (volume/volume) Tween-20 (PBS-Tween) and 5% (weight/volume) nonfat milk powder (Marvel, Premier brands, Spalding, UK). After washing with PBS-Tween, the nitrocellulose was incubated in undiluted MAb-containing supernatant (QUB Pa1 or QUB Pa2). The nitrocelluose then was washed in PBS-Tween before incubation with a goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma, Poole, UK). Bound MAbs were detected using an alkaline phosphatase conjugate substrate kit, containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Biorad Laboratories Ltd., Hercules, CA). The number of each subtype of P. acnes then was counted.

The incidence of bacterial contamination in each type of wound was compared using Fischer's exact test. A significance level of 0.05 was used.

RESULTS

Bacteria most frequently were cultured from skin samples (29.1%) and less frequently from wound tissue (21.5%) and wound wash (16.5%) (Fig 1). Staphylococcus aureus was detected in the skin, wound tissue and wound wash of one patient. All other Staphylococcus spp identified were coagulase-negative staphylococci (CoNS). We found no association between wound type (lumbar or nonlumbar) and either detection of or particular bacteria in the skin samples or wound wash using agar. We also found no association between wound type and the presence of P. acnes. Staphylococcus spp was detected more frequently in nonlumbar wounds than other sites ($p = 0.004$).

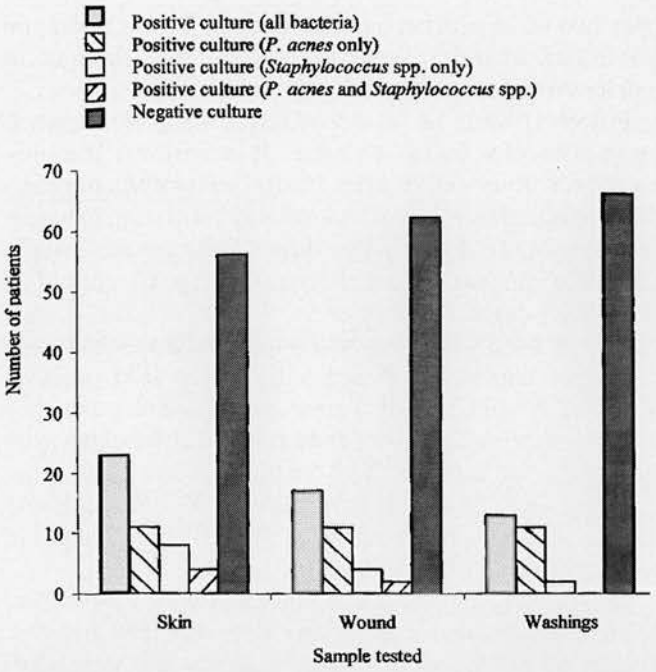

Fig 1. A histogram shows the bacteria detected in surgical specimens (skin, wound tissue, and wound washings) retrieved from patients having spinal surgery.

Seventeen (25.0%) of patients having primary surgery had positive cultures from wound tissue and 13 (19.1%) had positive cultures from wound washings. No patients having revision surgery had positive cultures from wound tissue or wound wash. This also was the case for the patient of whom the revision status was not known. There was no significant difference between revision status and positive culture from tissue or positive culture from wound wash.

Nine (11.4%) broth samples from the 79 patients had a positive culture. Propionibacterium acnes was cultured in eight (88.9%) of positive broth cultures and Staphylococcus spp was cultured in the remaining broth (11.1%). No enrichment broth contained both P. acnes and Staphylococcus spp. Enrichment broth was 57.7% specific and 96.1% sensitive for the detection of P. acnes and 25.0% sensitive and 99.0% specific for the detection of Staphylococcus spp.

The pure cultures isolated from 14 patients who were culture positive for P. acnes in the skin and other areas of the wound including 10 lumbar wounds, three anterior cervical wounds, and one abdominal wound, were typed using monoclonal antibodies specific for Type 1 and Type 2 P. acnes. The mean number of P. acnes colonies tested per patient was 11 colonies (range, 2–18 colonies per patient). In eight patients, only Type 1 P. acnes was detected and in four patients, only Type 2 P. acnes was detected.

Only two of 14 patients had a mixture of Type 1 and Type 2 P. acnes. Mixed P. acnes populations were detected in one lumbar and one anterior cervical wound.

Fifteen (19.0%) of 79 wound tissue samples examined using IFM had visible bacteria. P. acnes was the only bacterium observed in nine (60%) immunofluorescence positive samples. P. acnes was detected with Staphylococcus spp using IFM in a further three (20%) samples. Staphylococcus spp was the only bacterium detected using IFM in 3 (20%) cases.

Five of 14 (35.7%) wound tissue samples that had positive agar cultures for P. acnes also were IFM positive. Seven of 65 (89.2%) of wound tissue samples that had negative agar culture were IFM positive. Compared with agar culture of bacteria, IFM for the detection of P. acnes in this setting had a sensitivity value of 35.7%, specificity 89.2%, positive predictive value (PPV) 41.6%, negative predictive value (NPV) 86.6%.

One of 10 (10%) wound samples that were agar-culture positive for Staphylococcus spp also was IFM positive. Five of 69 (7.2%) wound tissue samples that were agar-culture negative were IFM positive. Immunofluorescence microscopy had a sensitivity value of 10%, specificity 92.8%, PPV 16.6%, and NPV 87.7%.

When bacteria were detected, they typically appeared as single cells (Fig 2) using fluorescence microscopy whereas bacteria detected in cases of prosthetic hip loosening appeared as large aggregates (Fig 3).

In the 10 healthy volunteers the area of skin most heavily colonized by P. acnes was the neck followed by the forehead, the lumbar spine, the abdomen, the chest, and the hip (Table 1). The forehead, neck and lumbar spine contained approximately 10^2 to 10^3 as many bacteria as the other sites. The lumbar spine had 4×10^2 as many P. acnes per cm^2 as the hip. Colonies were lifted from the agar onto nitrocellulose and reacted with either Type 1 or Type 2 monoclonal antibodies. In all sites sampled, P. acnes was detected most frequently in mixed Type 1 and Type 2 populations (Fig 4). The neck and the hip were the sites with the highest number of mixed populations. The forehead was the site most likely to contain only Type 1 P. acnes. The chest was the only site to contain an exclusively Type 2 P. acnes population.

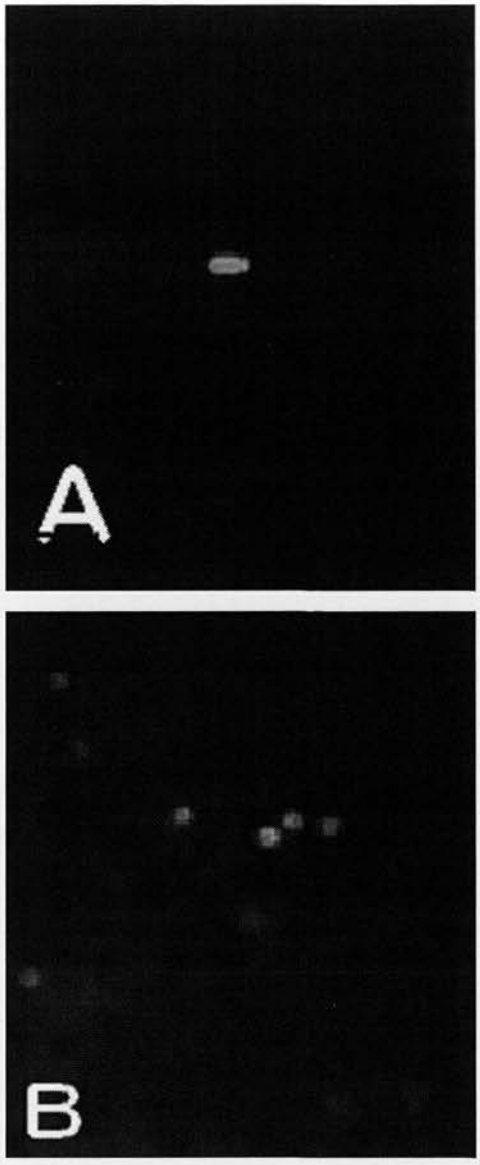

Fig 2A–B. Immunofluorescence micrographs show (A) P. acnes and (B) Staphylococcus spp detected in a contaminated spinal wound.

DISCUSSION

The purpose of this study was to determine the incidence of intraoperative wound contamination during spinal surgery and to identify the most likely source of contaminating organisms. We show that wound contamination during spinal surgery occurs relatively frequently by bacteria that form part of the normal skin microbiota. To date, there has been no formal examination of the incidence of wound contamination during spinal surgery. Other authors also have shown the presence of normal skin microbiota in surgical wounds. Padgett et al[18] found that 30% of revision hip arthroplasty wounds had positive bacterial cultures. The predominant organisms identified were principally S. epidermidis and P. acnes, as was the case in the current study. Dietz et al,[7] who sampled clean orthopaedic wounds (excluding total joint replacements) for the presence of bacteria, found that as many as 58% of wound samples had positive cultures and that the predominant bacteria isolated were also skin microbiota. Wollinsky et al[25] had similar

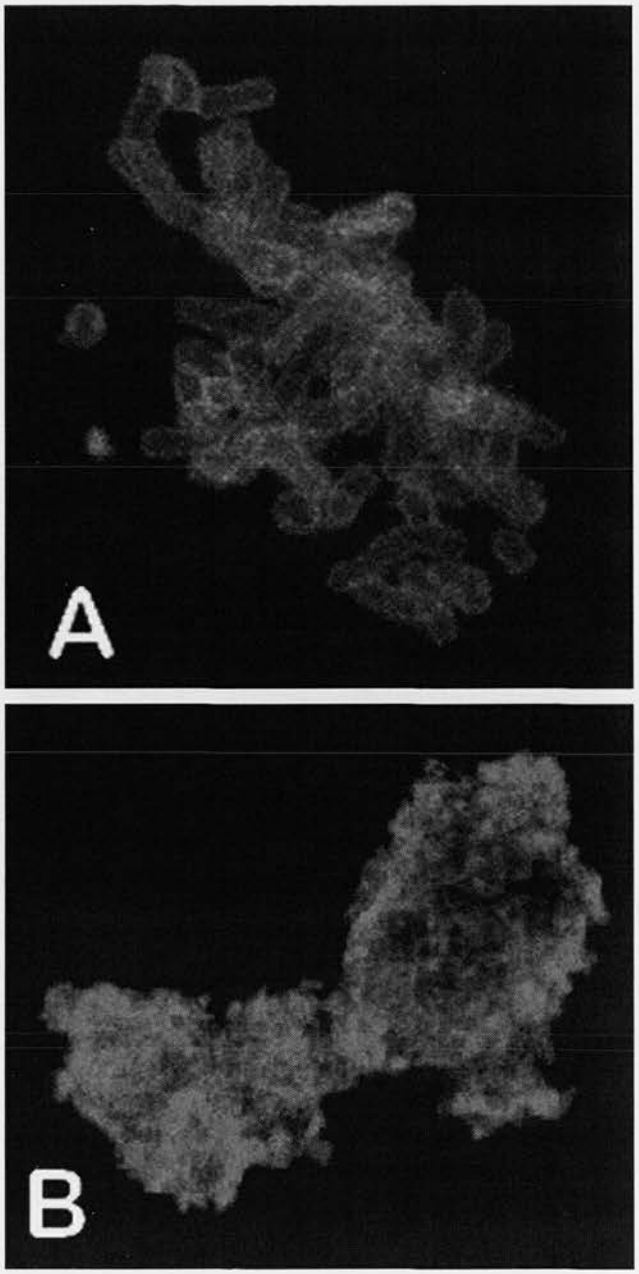

Fig 3A–B. Immunofluorescence micrographs show (A) P. acnes and (B) Staphylococcus spp detected in material dislodged by ultrasound from a failed hip replacement prosthesis (note large aggregates).

findings when they salvaged intraoperative blood from the surgical wounds was screened for the presence of bacteria. The incidence of positive cultures from wound tissue samples in the current study is slightly lower than that described in the literature (Table 2). This perhaps is explained by the fact that the majority of patients in the current study received antibiotics before samples were taken whereas no antibiotics were administered before samples were taken in the other studies. Stirling et al[22] make no reference to antibiotic prophylaxes in their study. The studies by Padgett et al,[18] Wollinsky et al,[25] and Dietz et al[7] show that CoNS are identified more frequently from orthopaedic wounds than P. acnes.[7,18,25] In the current study P. acnes was detected more frequently than CoNS. Stirling et al[22] also detected P. acnes more frequently from spinal surgical specimens and in the study by Dietz et al.[7] P. acnes was identified as frequently as CoNS when only spinal wounds are considered.[7,22] The current study did not identify previous spinal injection as a risk factor for positive culture from a wound. This also was the finding of Stirling et al.[22] Broth enrichment culture in our experience did not improve the detection of bacteria from surgical specimens. Broth enrichment often is used to improve the detection of bacteria. Dietz et al[7] demonstrated that enrichment improved bacterial detection by 15%.[7] It is possible in the current study that agar culture detection was more sensitive than broth enrichment because of the strict adherence to anaerobic conditions during transportation of samples, or the enrichment broth used might not be an ideal medium for the bacteria present in samples.

There were high numbers of cutaneous P. acnes particularly on the forehead, neck and lumbar spine area the skin of healthy volunteers. McGinley et al,[15] in a study of the regional variations on cutaneous propionibacteria, also found also found high levels of P. acnes in the forehead and other sebum rich areas.[15] To date no authors have examined specifically the phenotypes or numbers of P. acnes areas used to site orthopaedic incisions.

Bacterial contamination of the surgical wound is thought to occur because of aerial contamination of the wound,[4] or contamination from the patients own skin. Using fluorescence microscopy to identify the phenotypes of P. acnes found in operative samples we hoped this would enable the determination of origin of the P. acnes detected.

When fluorescence microscopy was compared with the colony lift method it was noted that only one of 10 (10%) lumbar wounds contained Type 1 and Type 2 P. acnes. This was in contrast with the P. acnes populations detected in unprepared lumbar skin from healthy volunteers, 7 of 10 (70%) who had a mixture of Type 1 and Type 2 P. acnes. The source of the P. acnes identified in the patient's wound is most likely to be the patient's own skin and this is supported by the fact that that the P. acnes phenotype identified in the surgical wound was the same as the P. acnes phenotype identified from the operative skin sample. Although the sample sizes are relatively small and the methods used are not directly comparable, it is possible that antiseptic solution may alter the natural balance of the P. acnes population in normal skin. Further work into the

TABLE 1. Comparison of P. acnes Populations at Different Skin Sites in Healthy Volunteers*

Subject Number	Age (years)	Sex (M/F)	Forehead (cfu/cm^2)	Neck (cfu/cm^2)	Chest (cfu/cm^2)	Abdomen (cfu/cm^2)	Lumbar Spine (cfu/cm^2)	Hip (cfu/cm^2)
1	48	F	3.6×10^5	3.0×10^5	1.0×10^3	2.3×10^3	1.8×10^4	8.4×10^2
2	31	M	2.3×10^5	8.5×10^5	6.5×10^2	1.2×10^2	1.0×10^5	5.0×10^0
3	27	F	6.8×10^3	1.1×10^2	7.2×10^0	6.0×10^1	0	1.0×10^0
4	21	F	2.0×10^3	2.1×10^2	1.1×10^1	1.5×10^1	4.3×10^2	1.2×10^2
5	12	F	6.9×10^2	6.5×10^2	1.6×10^3	7.6×10^1	1.0×10^2	6.2×10^1
6	34	M	2.7×10^3	2.8×10^5	4.5×10^2	2.7×10^2	1.0×10^2	9.0×10^0
7	30	M	3.7×10^4	3.1×10^3	4.0×10^0	3.7×10^3	3.3×10^4	9.0×10^0
8	27	F	6.8×10^3	9.0×10^2	4.0×10^0	3.0×10^1	0	1.0×10^0
9	28	M	4.0×10^4	2.3×10^5	1.9×10^2	2.0×10^1	2.8×10^5	5.2×10^1
10	18	F	2.8×10^4	1.2×10^1	1.0×10^0	9.0×10^0	3.0×10^0	1.0×10^0
Mean	27.6		7.2×10^4	1.7×10^4	3.3×10^2	6.6×10^2	4.4×10^4	1.1×10^2

*95% confidence intervals of colony counts fell within 1 log of stated values.

M = male; F = female; cfu = colony-forming units

possible resistance of the different P. acnes phenotypes to antiseptics is required.

It is known that antiseptic solutions used in surgery reduce the total bacterial count of the skin surface; however, they do not render the skin or wounds sterile.[1,12,17] It seems that P. acnes, which is less aerotolerant than the CoNS and usually is found in the depths of the follicle,[3] escapes the effects of an antiseptic as do some of the CoNS. When skin is incised, the bleeding skin edges facilitate the transport of bacteria from the follicles into the wound. Our results confirm the presumption because P. acnes was detected more frequently than Staphylococcus spp in surgical wounds. We also showed that the wound tissue and wound washings were positive for bacterial culture less frequently than skin. The interpretation of culture results from any spinal surgical wound must be treated with caution because 29.1% of wound skin was culture positive for P. acnes or CoNS. Staphylococcus aureus does not seem to cause substantial bacterial contamination of surgical wounds; authors of other studies failed to detect it at all.[7,25]

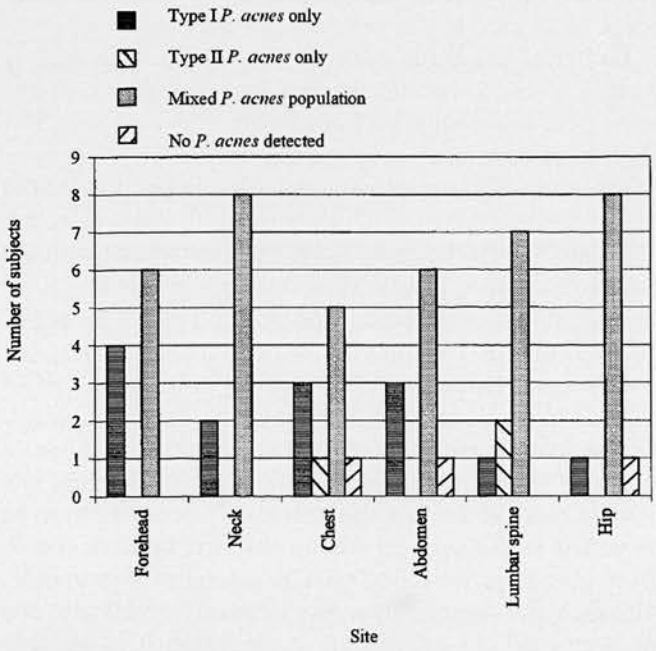

Fig 4. The number of P. acnes colonies detected at different sites in healthy volunteers is shown.

In contrast with the detection of biofilm-associated prosthetic joint infection, in the current wound study, IFM did not increase the chance of detection of bacteria when compared with culture.[23] In experiments with pure cultures in which total viable count (TVC) was compared with the limit of detection by IFM, only one or two bacteria were observed on a microscope slide well to which a 20 μL drop containing approximately 400 cfu/mL of P. acnes had been applied. This suggests that the bacteria observed in association with prosthesis biofilm are nonculturable whereas those in the wound study are viable and are present in much lower numbers. The majority of culture-positive clinical samples analyzed had fewer cfu/mL, and this explains why IFM often was negative.

Immunofluorescence microscopy has proven to be a useful tool in the detection of bacteria from failed hip prosthesis.[23] Most importantly, IFM permits observation of bacteria. Large aggregates are highly suggestive of bacterial biofilm-related infection; however, single bacterial cells suggest intraoperative contamination. In the contaminated wounds we studied, bacteria typically were detected as single cells (Fig 3); however, in cases of prosthetic hip loosening, large aggregates are found (Fig 4), and this would suggest biofilm formation.

TABLE 2. Comparison of Bacteria Isolated from Orthopaedic Wounds

Study	Site	Number of Patients Sampled	Number of Patients with Positive Cultures (%)	Percentage of Positive Cultures for P. acnes	Percentage of Positive Cultures for Staphylococcus spp
Padgett et al[18]	Hip	138	42 (30.4%)	23.6%	56.3%
Dietz et al[7]	Various*	40	23 (57.5%)	24.2%	57.6%
Dietz et al[7]†	Spine	12	7 (58.3%)	44.4%	44.4%
Stirling et al[22]	Spine	50	19 (38.0%)	84.2%	10.5%
Current Study‡	Spine	79	17 (21.5%)	64.7%	23.5%

*Various = extremity, pelvis, spine.

†A subset of 12 spine wounds out of the larger group of 40 wounds is considered.

‡Wound tissue sample culture results expressed.

Bacterial contamination of spinal surgical wounds occurs relatively frequently. This almost certainly reflects the high numbers of resident skin microbiota in the areas where the surgical incision is made. We suggest that P. acnes cultured from spinal surgery wounds originates from the skin and not from other sources. Bacterial culture using agar and enrichment techniques do not help distinguish bacterial contamination from infection. Immunofluorescence microscopy is potentially a valuable tool in distinguishing between contamination and infection because it permits direct observation of bacterial cells.

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CAMP factor homologues in *Propionibacterium acnes*: a new protein family differentially expressed by types I and II

Susanna Valanne,[1]† Andrew McDowell,[1] Gordon Ramage,[1]‡ Michael M. Tunney,[1]§ Gisli G. Einarsson,[1] Seamus O'Hagan,[1]|| G. Brian Wisdom,[2] Derek Fairley,[3] Ajay Bhatia,[4] Jean-Francois Maisonneuve,[4] Michael Lodes,[4] David H. Persing[4] and Sheila Patrick[1]

Correspondence
Sheila Patrick
s.patrick@qub.ac.uk

[1]Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Grosvenor Road, Belfast BT12 6BN, UK

[2]School of Biology and Biochemistry, Medical Biology Centre, 97 Lisburn Road, Queen's University, Belfast BT9 7BL, UK

[3]QUESTOR Centre, Queen's University, David Keir Building, Stranmillis Road, Belfast BT9 5AG, UK

[4]Corixa Corporation, Infectious Disease Institute, Seattle, WA 98104, USA

Analysis of the draft genome sequence of the opportunistic pathogen *Propionibacterium acnes* type strain NCTC 737 (=ATCC 6919) revealed five genes with sequence identity to the co-haemolytic Christie–Atkins–Munch-Peterson (CAMP) factor of *Streptococcus agalactiae*. The predicted molecular masses for the expressed proteins ranged from 28 to 30 kDa. The genes were present in each of the three recently identified *recA*-based phylogenetic groupings of *P. acnes* (IA, IB and II), as assessed by PCR amplification. Conserved differences in CAMP factor gene sequences between these three groups were also consistent with their previous phylogenetic designations. All type IA, IB and II isolates were positive for the co-haemolytic reaction on sheep blood agar. Immunoblotting and silver staining of SDS-PAGE gels, however, revealed differential protein expression of CAMP factors amongst the different groups. Type IB and II isolates produced an abundance of CAMP factor 1, detectable by specific antibody labelling and silver staining of SDS-PAGE gels. In contrast, abundant CAMP factor production was lacking in type IA isolates, although larger amounts of CAMP factor 2 were detectable by immunoblotting compared with type II isolates. While the potential role of the abundant CAMP factor 1 in host colonization or virulence remains to be determined, it should be noted that the type strain of *P. acnes* used in much of the published literature is a type IA isolate and is, therefore, lacking in this attribute.

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†**Present address:** Institute of Medical Technology, University of Tampere, Finland.

‡**Present address:** Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, UK.

§**Present address:** School of Pharmacy, Queen's University, Belfast, UK.

||**Present address:** Withers Orthopaedic Unit, Musgrave Park Hospital, Belfast, UK.

Abbreviations: CAMP, Christie–Atkins–Munch-Peterson; IFM, immunofluorescence microscopy.

The GenBank/EMBL/DDBJ accession numbers for the CAMP factor sequences of *P. acnes* strains NCTC 737 (IA), KPA171202 (IB), NCTC 10390 (II) and SG2 (II) are given in Table 3.

INTRODUCTION

As a member of the resident human microbiota, the Gram-positive anaerobic coryneform bacterium *Propionibacterium acnes* is found predominantly in the sebaceous gland-rich areas of the skin in adults (Eady & Ingham, 1994). It can, however, also be isolated from the conjunctiva, the external ear canal, the mouth, the upper respiratory tract and, in some individuals, the intestine (Funke *et al.*, 1997). It accounts for approximately half of the total skin microbiota (Tancrede, 1992), with an estimated density of 10^2 to 10^{5-6} cm^{-2} (Leyden *et al.*, 1998; McGinley *et al.*, 1978). In most people it outnumbers coagulase-negative *Staphylococcus* spp. (CoNS) on the skin by 10- to 100-fold (Eady

& Ingham, 1994). *P. acnes* is a well-recognized opportunistic pathogen, especially in relation to medical implants such as central nervous system shunts (Brook & Frazier, 1991), silicone implants (Ahn *et al.*, 1996) and prosthetic hip joints, where it is recovered as frequently as CoNS (Tunney *et al.*, 1998, 1999b). It is also responsible for ocular and periocular infections and endophthalmitis (Aldave *et al.*, 1999; Clark *et al.*, 1999) and has been implicated in periodontal and dental infections (Le Goff *et al.*, 1997). Indeed, dental probing and treatment can lead to the dissemination of *P. acnes* in the bloodstream (Debelian *et al.*, 1992), which is a recognized cause of endocarditis in relation to damaged or prosthetic heart valves. *P. acnes* is also considered to play a crucial role in inflammatory acne (Eady & Ingham, 1994), since antimicrobial therapy directed against *P. acnes* results in improvement, while the development of antibiotic resistance in *P. acnes* is associated with relapse (Leyden *et al.*, 1998). The common form of acne, known as acne vulgaris, affects up to 80 % of the population at some time in their lives, making it the most common skin infection. There is also a strong association between severe forms of acne and joint pain, inflammation of the bone (osteitis) and arthritis. In patients suffering from this condition, known as SAPHO (synovitis, acne, pustulosis, hyperostosis and osteitis) syndrome, isolates of *P. acnes* have been recovered from bone biopsy samples, as well as synovial fluid and tissue (Schaeverbeke *et al.*, 1998). Immunologists have long recognized and exploited the potent adjuvant activity of *P. acnes* and utilized it in models of inflammation, although many continue to refer to it as '*Corynebacterium parvum*' (Tasaka *et al.*, 1996).

Studies by Johnson & Cummins (1972) first revealed two distinct phenotypes of *P. acnes*, known as types I and II, based on serological agglutination tests and cell-wall sugar analysis. Recently, *recA*-based sequence analysis has revealed that *P. acnes* types I and II represent phylogenetically distinct groups (McDowell *et al.*, 2005). Furthermore, a small subgroup of phylogenetically distinct type I strains with atypical mAb labelling characteristics, which we now designate type IB to distinguish them from other type I strains, designated type IA, were also described. The observation that the phenotypic differences between strains of the various *P. acnes* types reflect deeper differences in their phylogeny raises the possibility that they may also display variation in their expression of putative virulence factors.

P. acnes produces a co-haemolytic reaction with both sheep and human erythrocytes (Choudhury, 1978) similar to the Christie–Atkins–Munch-Petersen (CAMP) reaction first demonstrated in 1944 (Christie *et al.*, 1944). The CAMP reaction describes the synergistic haemolysis of sheep erythrocytes by the CAMP factor from *Streptococcus agalactiae* and the β-toxin (sphingomyelinase C) from *Staphylococcus aureus*, with the CAMP factor demonstrating non-enzymic affinity for ceramide (Bernheimer *et al.*, 1979). Examination of sphingomyelinase-treated sheep erythrocytes has revealed the formation of discrete membrane pores by recombinant *Streptococcus agalactiae* CAMP factor (Lang & Palmer, 2003). In addition to the extensive study of the CAMP factor of *Streptococcus agalactiae* (Bernheimer *et al.*, 1979; Brown *et al.*, 1974; Jurgens *et al.*, 1985, 1987; Ruhlmann *et al.*, 1988; Skalka *et al.*, 1980), a number of other Gram-positive and Gram-negative bacteria are known to produce a positive CAMP reaction, including *Pasteurella haemolytica* (Fraser, 1962), *Aeromonas* species (Figura & Guglielmetti, 1987), some *Vibrio* species (Kohler, 1988) and group G streptococci (Soedermanto & Lammler, 1996). Some of these species can also use phospholipase C (α-toxin) from *Clostridium perfringens* or phospholipase D from *Corynebacterium pseudotuberculosis* as a co-factor for haemolysis in addition to the *Staphylococcus aureus* β-toxin (Frey *et al.*, 1989). The CAMP factor genes of *Actinobacillus pleuropneumoniae* and *Streptococcus uberis* have also been identified, cloned and expressed in *Escherichia coli* (Frey *et al.*, 1989; Jiang *et al.*, 1996).

The precise role of the CAMP molecule in bacterial virulence remains unclear. It is likely that the co-haemolytic reaction represents a laboratory phenotype, or epiphenomenon, that is convenient for CAMP factor detection, but which may not be directly related to the role of the molecule in colonization and pathogenesis. The CAMP factor from *Streptococcus agalactiae* binds to the Fc region of IgG and IgM molecules, similar to the binding of IgG by *Staphylococcus aureus* protein A (Jurgens *et al.*, 1987), and partial amino acid sequence similarity between the CAMP factor protein of *Streptococcus agalactiae* and *Staphylococcus aureus* protein A has been demonstrated (Ruhlmann *et al.*, 1988). We now present evidence of differences amongst *P. acnes* types IA, IB and II in the expression of proteins with sequence similarity to the CAMP co-haemolysin.

METHODS

Bacterial isolates, media and culture conditions. The following reference strains were from the National Collection of Type Cultures (NCTC, Colindale, UK), the American Type Culture Collection (ATCC, Manassas, VA, USA) and the National Collections of Industrial, Marine and Food Bacteria (NCIMB, Aberdeen, UK): *P. acnes* NCTC 737 (=ATCC 6919) and NCTC 10390, *Propionibacterium granulosum* NCTC 10387, *Staphylococcus aureus* ATCC 25923, *Actinomyces israelii* NCTC 8047, *Actinomyces naeslundii* NCTC 10301, *Micrococcus luteus* NCIMB 13267 (formerly Fleming strain 2665) and *Bacteroides fragilis* NCTC 9343. A total of 112 isolates of *P. acnes* were examined. Sixty-seven isolates were recovered from failed prosthetic hip joints and associated bone, tissue and skin samples removed from patients attending Musgrave Park Orthopaedic Hospital, Belfast, as detailed previously (Tunney *et al.*, 1998, 1999b). In addition, a further group of *P. acnes* isolates recovered from tissue samples removed during revision arthroplasty in Sweden ($n=18$), as well as acne ($n=19$) and dental ($n=8$) infections, were kind gifts. Isolates of *Propionibacterium acidipropionici, Streptococcus agalactiae, Staphylococcus epidermidis, Streptococcus lactis, Streptococcus equi, Streptococcus sanguis, Streptococcus salivarius, E. coli* DH5α and *Propionibacterium avidum* were obtained from the Department of Microbiology and Immunobiology culture collection, Queen's University, Belfast.

Bacterial culture. All anaerobic strains were grown on anaerobic blood agar (ABA) (CM0972; Oxoid) or in brain heart infusion (BHI) (CM225; Oxoid) broth. Cultures were incubated at 37 °C in an anaerobic cabinet (MACS MG 1000; Don Whitley Scientific), in an atmosphere of 80 % N_2, 10 % CO_2 and 10 % H_2. All *Staphylococcus* and *Streptococcus* strains were also grown at 37 °C on blood agar (BA). *E. coli* DH5α was grown aerobically on Luria–Bertani agar plates at 37 °C. Isolates of *P. acnes* were routinely identified using the API 20A multitest identification system (bioMérieux) in accordance with the manufacturer's instructions.

Co-haemolysis assay. Co-haemolytic activity was monitored by a modification of the classical co-haemolysis reaction on sheep BA plates as originally described (Christie *et al.*, 1944). Briefly, the *Staphylococcus aureus* strain ATCC 25923 was streaked vertically onto sheep BA and the test strain was then streaked horizontally outwards from either side, starting close to, but not touching, the *Staphylococcus aureus* streak. Plates were incubated anaerobically at 37 °C for 48 h. A butterfly-shaped zone of lysis at the junction of the streaks was caused by the co-effect of diffusing *Staphylococcus aureus* β-toxin and co-haemolytic factor.

Production of mAb and rabbit polyclonal antisera. The mAb QUBPa4 was generated using the protocol described previously (Harlow & Lane, 1988; Tunney *et al.*, 1999a). Four BALB/c mice were immunized with killed whole cells (10^8 c.f.u. ml^{-1}) of *P. acnes*. The hybridoma cell line producing QUBPa4 was then cloned by limiting dilution (Harlow & Lane, 1988).

Rabbit polyclonal antisera were prepared against the five CAMP proteins using recombinant products expressed in *E. coli*. CAMP genes were amplified from *P. acnes* NCTC 737 genomic DNA and subcloned into the plasmid vector pET17b (Stratagene). Ligation products were first transformed into *E. coli* XL-1 Blue competent cells (Stratagene) and the plasmid DNA isolated from XL-1 Blue transformants was subsequently transformed into *E. coli* BL21 (DE3) pLysE or pLysS host cells (Novagen). The recombinant proteins were expressed in *E. coli* with a poly-histidine tag at the N terminus and were purified from IPTG-induced batch cultures, in the presence of 8 M urea, by affinity chromatography using the one-step QIAexpress Ni-NTA agarose matrix (Qiagen). Purity of the recombinant proteins was assessed by SDS-PAGE, followed by Coomassie brilliant blue staining and N-terminal sequencing using Edman chemistry with a Procise 494 protein sequencer (Perkin-Elmer Applied Biosystems). All recombinant proteins were assayed for endotoxin contamination using the *Limulus* amoebocyte assay (BioWhittaker) and shown to contain less than 50 endotoxin units mg^{-1}. Polyclonal rabbit antiserum was raised against all recombinant proteins by injecting New Zealand white rabbits (R&R rabbitry) with 200 μg purified recombinant protein in incomplete Freund's adjuvant (IFA) plus 100 μg muramyl dipeptide (Sigma). Serum was collected following two subsequent boosts separated by 3 weeks with 100 μg protein in IFA. As a result of problems with stability of the CAMP factor 1 and 5 recombinant proteins, only an N-terminal fragment of the CAMP 1 protein and N-terminal and C-terminal fragments of the CAMP 5 protein were used for immunization.

Patient serum. Serum was prepared from venous blood (10 ml) taken pre-operatively from patients about to undergo either primary or revision total hip arthroplasty at Musgrave Park Hospital, Belfast, and from acne patients attending a Dermatology outpatients clinic in the Royal Victoria Hospital, Belfast. These procedures were approved by the local ethical committee and all patients gave full consent.

Immunofluorescence microscopy (IFM). IFM was carried out as described previously (Patrick *et al.*, 1995) with minor modification. Briefly, bacterial cultures were grown on ABA or BA and a suspension of 10^8 c.f.u. ml^{-1} was prepared in 0·01 M PBS (0·15 M NaCl, 0·0075 M Na_2HPO_4, 0·0025 M $NaH_2PO_4.2H_2O$; pH 7·4). Samples (10 μl) were then applied to multiwell slides, air-dried and fixed in 100 % methanol for 10 min at −20 °C. Undiluted hybridoma cell culture supernatant containing mAb QUBPa4 (30 μl) was added to each well of the slides and incubated for 45 min at 37 °C. After washing in 0·01 M PBS for 20 min at room temperature, a 1 : 100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) in PBS containing 0·1 % (w/v) Evans Blue (Merck Sharp & Dome) counterstain (30 μl) was applied to each well and incubated for 45 min at 37 °C. Control wells in which primary antibody was replaced with PBS were routinely included to monitor non-specific binding of the secondary antibody. Slides were then washed and mounted in glycerol-PBS, containing an anti-photobleaching agent (Citifluor; Agar Scientific), and examined using a Leitz Dialux 20 fluorescence microscope.

Preparation of bacterial extracts. To obtain efficient extraction and reproducible recovery of loosely cell-associated and secreted CAMP factor 1, standardized whole cell bacterial preparations of 1×10^{10} c.f.u. ml^{-1} were obtained by suspending a culture grown for 6 days on ABA directly into PBS, such that a 1 : 100 dilution had an OD_{600} of 0·3. Cells were then disrupted by ultrasound (Soniprep 150; 26 μm amplitude) for 5 min at 4 °C. The sonicated suspension was brought to room temperature and Tween 20 (Bio-Rad) was added to a final concentration of 2 mM. The sample was then centrifuged and the resulting pellet discarded. Sodium azide was added to the supernatant (final concentration 0·02 % v/v) before storage at −20 °C.

To investigate the presence of secreted CAMP factor 1 protein in culture supernatant, bacteria were grown in BHI broth for 24 h and then centrifuged at 2370 ***g*** for 30 min (Mistral; MSE) at room temperature and the supernatant retained. An equal volume of ice-cold ethanol was then added to the supernatant followed by overnight incubation at 4 °C. The precipitated material was recovered by centrifugation at 12 000 ***g*** for 30 min (Sorvall) and the pellet was resuspended in 500 μl distilled water.

SDS-PAGE and immunoblotting. Bacterial extracts, prepared as detailed above, were analysed using 9 % SDS-PAGE gels (Laemmli, 1970) and the resolved proteins were visualized using a silver staining kit (Amersham Pharmacia Biotech). To afford standardization and comparison of different *P. acnes* isolates for CAMP factor 1 expression, the colour development was carried out for between 110 and 120 s. Gels were washed three times for 5 min each in distilled water, placed in a drying solution [30 % (v/v) ethanol, 5·3 % (v/v) glycerol] for two periods of 30 min and preserved between cellophane sheets. The gels were then photographed using a Kodak DC290 digital camera fitted on a Kodak EDAS290 gel imaging hood and images were analysed using Kodak IK image analysis software version 3.5. Known positive and negative strains were included in each experiment as internal controls and indicated that the experimental system was reproducible.

Immunoblotting was carried out as described previously with a minor modification (Patrick & Lutton, 1990). In brief, nitrocellulose was blocked with 0·01 M PBS containing 0·05 % (v/v) Tween 20 (PBST) and 5 % (w/v) non-fat milk powder (Marvel; Premier brands). After washing with PBST, the nitrocellulose was incubated in undiluted mAb supernatant or an appropriate dilution of polyclonal antiserum in PBS. The nitrocellulose was then washed in PBST before incubation with alkaline phosphatase-conjugated goat anti-human IgG (H), anti-mouse IgG (H + L) or anti-rabbit IgG (H + L) (Sigma). Controls in which the primary antibody was replaced by PBS were routinely included to monitor non-specific binding of the secondary antibody.

Bound antibodies were detected using an alkaline phosphatase conjugate substrate kit containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Purification and analysis of CAMP factor 1 protein. The mAb QUBPa4 was purified using a HiTrap protein G column (Amersham Biosciences), concentrated by ultrafiltration and immobilized on CNBr-activated agarose (Sigma). The antibody gel was packed into a column and equilibrated with PBS. Extracts of ABA-grown *P. acnes* (isolate SG2), prepared as detailed above, were diluted in PBS and recycled through the column overnight. The bound antigen was released with 100 mM glycine/HCl buffer, pH 2·7, and immediately neutralized with 1 M Tris before storage at −20 °C. For trypsin digestion, the eluted fractions from the affinity chromatography column, containing approximately 120 μg purified protein, were pooled and dialysed against distilled water at 4 °C overnight. The sample was then lyophilized using a Speed-Vac (Savant), the precipitate dissolved in 20 μl 8 M urea, 0·4 M NH_4CO_3 and the pH adjusted to between 7·5 and 8. DTT (0·25 μmol) was added and the suspension was incubated at 50 °C for 15 min. After cooling to room temperature, iodoacetic acid (0·5 μmol) was added and the suspension was incubated at room temperature for a further 15 min. The final concentration of urea was then reduced to 2 M (final volume of digest 80 μl) by the addition of 50 μl distilled water. Trypsin (2·5 mg ml^{-1}; Roche) was added in a 1:25 ratio to the CAMP factor 1 protein (w/w) and the mixture was incubated at 37 °C for 24 h. Digestion was stopped by freezing the sample at −20 °C. Purified antigen and its fragments were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad). The bands were stained with Coomassie brilliant blue, excised and sequenced by Edman chemistry at the Babraham Institute (Cambridge, UK).

PCR amplification and sequencing. PCR was used to detect the five CAMP factor homologue genes in a selection of *P. acnes* strains. CAMP factor genes were amplified using primers directed to downstream and upstream flanking sequences of each ORF (based on the *P. acnes* NCTC 737 genome sequence), thus facilitating accurate sequence determination of the 5′ and 3′ ends of each ORF. Preparation of bacterial genomic DNA and PCR amplifications were carried out essentially as described previously (McDowell *et al.*, 2005). PCR samples contained 1 × PCR buffer, 200 μM of each dNTP (Amersham Pharmacia Biotech), 200 μM of each appropriate CAMP factor oligonucleotide primer (Table 1), 1·5 mM $MgCl_2$, 1·25 U Platinum *Taq* DNA polymerase (Invitrogen Life Technologies) and 2·5 μl bacterial lysate, in a total volume of 25 μl. Samples were initially heated at 95 °C for 3 min, followed by 35 cycles of 1 min at 95 °C, 30 s at the appropriate annealing temperature (Table 1) and 1 min at 72 °C. The PCR was completed with a final extension step at 72 °C for 10 min. A negative water control was included in all experiments. All PCR products were analysed as described before (McDowell *et al.*, 2005). Sequencing reactions were performed using ABI PRISM Ready Reaction Terminator cycle sequencing kits (Perkin Elmer Applied Biosystems) according to the manufacturer's instructions. Samples were analysed on an ABI PRISM 3100 DNA sequencer (Perkin Elmer Applied Biosystems).

Sequence and phylogenetic analyses. A draft sequence of the *P. acnes* NCTC 737 genome was commissioned from Genset (Evry, France) by Corixa Corporation (Seattle, WA, USA). A total of 26 million bases of DNA were sequenced, representing approximately 10 genome equivalents. An annotated database of ORFs was created using seven Genemark predictive models, as well as BLASTP, Psort, SignalP, Pfamm, InterProScan and other bioinformatic applications. Comparison with the *P. acnes* KPA171202 genome sequence (Bruggemann *et al.*, 2004) was carried out using the Artemis Comparison Tool (ACT; http://www.sanger.ac.uk/Software/ACT/). Consensus trees showing protein and nucleotide sequence relationships were generated using the Data Analysis in Molecular Biology and Evolution software (DAMBE; http://aix1.uottawa.ca/~xxia/software/software.htm). Multiple sequence alignments were performed using the CLUSTAL W algorithm (Thompson *et al.*, 1994) and exported into the DAMBE program. For nucleotide analysis, consensus trees were constructed using the maximum-parsimony method and the neighbour-joining method using the Jukes–Cantor-based algorithm. Sequence input order was randomized and bootstrapping resampling statistics were performed using 100 datasets for each analysis.

RESULTS

Identification of multiple genes with CAMP factor sequence identity

Analysis of the draft genome sequence of the *P. acnes* type IA strain NCTC 737 (=ATCC 6919) (property of Corixa Corporation) identified five related genes, sited at different locations within the genome, with similarity to the *Streptococcus agalactiae* co-haemolysin or CAMP factor protein (GenBank accession no. X72754.1) originally described by Christie, Atkins and Munch-Petersen in 1944 (Christie *et al.*, 1944). The resulting CAMP factor protein sequences had predicted molecular masses that ranged from approximately 28 to 30 kDa. All five genes have a putative N-terminal signal peptide cleavage site (Table 2).

Table 1. PCR primers used in this study for amplification of *P. acnes* CAMP factor genes

Target	Primer	Sequence (5′–3′)	Position	Annealing temperature (°C)	Product size (bp)
CAMP 1	C1-F	GCTTGCAGTTGCGAGCAATTGTTC	−44 to −21	60	946
	C1-R	CCCATGCCGTAAATGATTTCGATG	902 to 879		
CAMP 2	C2-F	GTCGTAGCCATACACCACACG	−172 to −152	60	1015
	C2-R	GCACCGAGTGTTGATGTCAATTAGC	843 to 819		
CAMP 3	C3-F	AATCGTGGCGGGGAGGTTAGTA	−102 to −81	62	1000
	C3-R	GACACGTCAATAGGGGAGAAGAAG	898 to 875		
CAMP 4	C4-F	CAGATCGACCAACGCTTTAGG	−139 to −119	58	1039
	C4-R	CCACCACAGGCACGGATTGA	900 to 881		
CAMP 5	C5-F	CCACGCCATGAGCTAAGGACAG	−246 to −225	62	1178
	C5-R	TGAACTAGACCGCGGCAAACATT	932 to 910		

Table 2. Characteristics of the predicted *P. acnes* CAMP factor proteins of NCTC 737, KPA 171202 and NCTC 10390

Strains belong to types IA (NCTC 737), IB (KPA171202) and II (NCTC 10390).

Protein	Calculated molecular mass (Da)*			Amino acids	Theoretical pI			Putative signal sequence cleavage site (positions)
	NCTC 737	KPA 171202	NCTC 10390		NCTC 737	KPA 171202	NCTC 10390	
CAMP 1	30391	30376	30160	285	9·77	9·88	9·74	AHA–AP (27–28)
CAMP 2	28538	28608	28673	267	9·52	9·61	9·52	AHA–VE (27–28)
CAMP 3	29042	29210	29159	271	9·50	9·67	9·59	AVA–AP (25–26)
CAMP 4	28245	28211	28273	267	9·50	9·50	9·50	AQA–SA (27–28)
CAMP 5	29855	29821	29905	281	9·68	9·68	9·61	AQA–AX† (27–28)

*Including signal sequence.
†V in type I; I in type II.

Construction of a bootstrapping consensus tree (using 100 bootstrapping replications) based on the different *P. acnes* CAMP factor protein sequences of NCTC 737, along with the CAMP factor sequences from *Streptococcus agalactiae* (GenBank accession no. NP_736433.1), *Streptococcus uberis* (accession no. AAA78910.1) and *Streptococcus pyogenes* (accession no. NP_802366.1), revealed that CAMP factor 3 was more distantly related to the other *P. acnes* CAMP factor proteins (Fig. 1). The *P. acnes* CAMP factors 1 and 5 were found to be more closely related to one another, as were CAMP factors 2 and 4.

Nucleotide sequence analysis of CAMP factor genes in *P. acnes* types I and II

The presence of all five CAMP factor genes in other strains of *P. acnes* type IA, as well as strains of type IB and type II, was revealed by PCR with primers designed to downstream and upstream flanking sequences of each CAMP factor ORF. PCR analysis of 12 isolates representative of *P. acnes* type IA (NCTC 737, RM1, PV37), type IB (CK17, LED2, RM9, W1392, W1998) and type II (NCTC 10390, SG2, RM4 and P24) resulted in PCR products of the correct size for all five CAMP factor genes, in all the isolates examined (data not shown). Nucleotide sequence analysis of all five CAMP factor genes from strains selected to represent type IA (NCTC 737) and type II (NCTC 10390 and SG2) was carried out (Table 3). These sequences were then compared with the CAMP factor gene sequences from the recently published genome sequence of *P. acnes* KPA171202 (Bruggemann *et al.*, 2004; accession no. AE017283), which we identified as a type IB strain based on *recA* and *tly* phylogenetic analysis. Consensus trees of the individual CAMP factor gene sequences from these strains were constructed using the maximum-parsimony and neighbour-joining methods. After 100 bootstrapping replications, the consensus trees derived using the two methods gave identical topologies. For CAMP factors 1, 2, 4 and 5, the phylogenetic relationships between the IA, IB and II strains were identical to that determined previously by *recA* and *tly* gene analysis (illustrated in Fig. 2a for CAMP factor 1). For CAMP 3, however, the consensus tree differed, as the type IA sequence was found to have a closer relationship to the type II sequence, with the type IB sequence being more distinct (Fig. 2b).

Comparison was also made between strains NCTC 737 (IA) and KPA171202 (IB) for nucleotide and amino acid

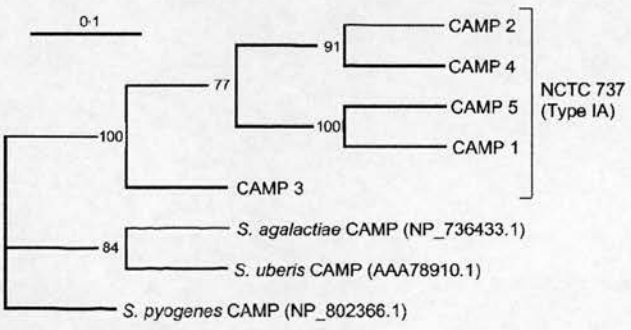

Fig. 1. Consensus tree illustrating the relationship between CAMP factor protein sequences from *P. acnes* NCTC 737 (type IA), *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus pyogenes*. Multiple sequence alignments were performed and the resulting phylogenetic tree was rooted with the *Streptococcus pyogenes* sequence. Bootstrapping resampling statistics were applied to the trees (100 datasets) and bootstrap values are shown at each node of the tree.

Table 3. GenBank accession numbers for CAMP factor sequences of *P. acnes* NCTC 737 (IA), KPA171202 (IB), NCTC 10390 (II) and SG2 (II)

Protein	NCTC 737	KPA171202	NCTC 10390	SG2
CAMP 1	AY527218	AAT83089	AY787764	AY787769
CAMP 2	AY726656	AAT82444	AY787765	AY787770
CAMP 3	AY726657	AAT83815	AY787766	AY787771
CAMP 4	AY726658	AAT82980	AY787767	AY787772
CAMP 5	AY726659	AAT82947	AY787768	AY787773

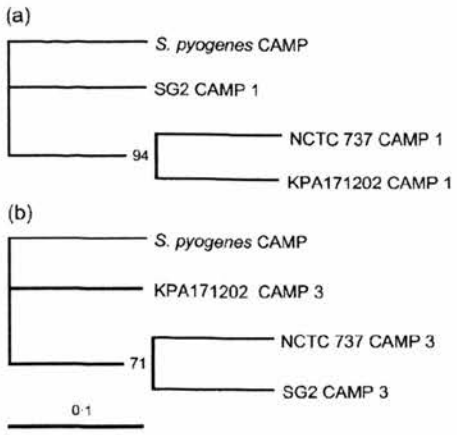

Fig. 2. Consensus trees illustrating the relationship between *P. acnes* types IA, IB and II CAMP factor genes. Multiple alignments of CAMP factor 1 (a) and CAMP factor 3 (b) gene sequences from strains NCTC 737 (type IA), KPA171202 (type IB) and SG2 (type II) were performed, along with the published CAMP sequence of *Streptococcus pyogenes*, which was used as an outgroup for the phylogenetic analysis. Consensus trees were constructed using the neighbour-joining method (with Jukes–Cantor-based algorithm). Bootstrapping resampling statistics were applied to the trees (100 datasets) and bootstrap values are shown at each node on the trees.

sequence differences in proteins putatively involved in the degradation of host molecules, as well as those thought to be cell-surface-associated. Analysis revealed variable numbers of nucleotide and amino acid sequence differences between the two strains (Table 4).

CAMP factor homologues of *P. acnes* and IgG-binding domains of protein A

As IgG binding by the *Streptococcus agalactiae* CAMP factor (Jurgens *et al.*, 1987), as well as partial amino acid sequence similarity of the molecule to protein A of *Staphylococcus aureus* (Ruhlmann *et al.*, 1988), has been demonstrated, sequence comparisons were made between the *P. acnes* CAMP factors and staphylococcal protein A. CLUSTAL W analyses identified a protein domain between positions 108 and 157 in CAMP factor 1 with 25 % or more identity to the IgG-binding domains E, B and C of *Staphylococcus aureus* protein A (Table 5). In addition, CAMP factor 3 showed approximately 25 % identity to domain A between positions 104 and 142. The CAMP factor of *Streptococcus agalactiae* was found to have considerably lower percentage identity.

Co-haemolytic (CAMP) reactivity of *P. acnes* types I and II

Fifty isolates of *P. acnes* (types IA, IB and II) recovered from failed prosthetic hip joints and associated bone and tissue samples, as well as skin and dental sources and strains of *Staphylococcus epidermidis*, *A. israelii*, *A. naeslundii* and *Streptococcus agalactiae*, were positive for the co-haemolytic CAMP reaction on sheep BA using a positive β-toxin-producing strain of *Staphylococcus aureus* (ATCC 25923). All *P. granulosum* strains were negative for the CAMP reaction, as were strains of *P. avidum*, *P. acidipropionici*,

Table 4. Percentage identity and number of nucleotide/amino acid differences between KPA171202 (type IB) and NCTC 737 (type IA) CAMP factors, selected putative host cell molecule-degrading factors and antigens

KPA171202 ORF	Putative function	Nucleotide sequence		Amino acid sequence	
		Differences (*n*)	Identity (%)	Differences (*n*)	Identity (%)
0687	CAMP factor	1	99·9	1	99·6
1198	CAMP factor	1	99·9	1	99·6
1231	CAMP factor	1	99·9	1	99·6
1340	CAMP factor	2	99·8	2	99·3
2108	CAMP factor	9	98·9	3	98·5
1396	Haemolysin	2	99·7	1	99·6
0685	Sialidase	2	99·9	0	100
1560	Sialidase	39	97·2	18	96·3
1569	Sialidase	56	98·6	19	98·6
1796	Triacyl glycerol lipase	37	96·4	11	96·5
2105	Triacyl glycerol lipase	9	99·1	6	98·2
1745	Phosphoesterase	3	99·9	2	99·8
0109	Myosin cross-reactive antigen	47	97·4	7	98·8
0453	GroEL	10	99·4	0	100
1955	Surface protein	57	96·9	27	95·6

Table 5. Comparison of predicted CAMP factor amino acid sequences of *P. acnes* type IA (NCTC 737) and type II (NCTC 10390) with the IgG-binding domains of *Staphylococcus aureus* protein A

Values are percentages of identity. Where type IA and II differ, the values are listed before (NCTC 737) and after (NCTC 10390) the solidus (/).

CAMP factor	*Staphylococcus aureus* protein A IgG-binding domain				
	E	D	A	B	C
1	25·0	23·2	17·6	28·0	30·0
2	12·0/14·0	14·9/15·7	15·7	10·9/15·7	13·5
3	19·2	19·3	25·6	19·1	17·0
4	15·4	16·7	14·5	13·0	15·7
5	18·4	11·1	14·0	15·8	22·0
Streptococcus agalactiae	12·2	11·8	11·5	13·5	15·4
Streptococcus uberis	14·3	18·0	17·5	14·8	16·7
Streptococcus pyogenes	15·4	14·8	19·6	17·6	17·6

M. luteus, *Streptococcus equi*, *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus lactis*, *E. coli* DH5α and *B. fragilis*.

Differences in CAMP factor protein expression between *P. acnes* types I and II

Expression of the five CAMP factor proteins by type I and II isolates was investigated by immunoblotting with rabbit polyclonal antisera raised against recombinant forms of each NCTC 737 CAMP factor protein, as well as a mouse mAb specific for CAMP factor 1. Representative immunoblots of NCTC 737 (type IA) and the prosthetic hip joint isolate SG2 (type II) are presented in Fig. 3. Labelled bands were observed in the correct region for the predicted molecular mass of the proteins, minus the signal sequence. Bands of lower molecular mass than that of the predicted secreted protein were sometimes observed and are believed to relate to degradation of a predicted labile N-terminal region. Isolates of *P. acnes* type II were found to produce large amounts of CAMP factor 1 compared with type IA strains. A corresponding abundant protein band was also observed on silver-stained SDS-PAGE gels of *P. acnes* type II, but was absent in type IA isolates (Fig. 4) and NCTC 10390 (type II; data not shown). Larger quantities of CAMP 2, however, were detectable in type IA isolates compared with type II by immunoblotting, although levels of the protein were still considerably less than CAMP factor 1 production (Fig. 3). Analysis of strains of *P. acnes* type IB by immunoblotting and silver staining of SDS-PAGE gels revealed a pattern of CAMP factor expression similar to that of type II organisms, with the production of large amounts of CAMP factor 1 and reduced expression of CAMP factor 2 compared with type IA strains (data not shown). Nucleotide sequences immediately upstream of the CAMP genes were compared to determine whether sequence differences in these regions could be influencing expression. Analysis revealed conservation of putative core Shine–Dalgarno sequences amongst the different *P. acnes* types for CAMP factors 1, 2, 3 and 5, but one base difference in the CAMP factor 1 Shine–Dalgarno sequence of NCTC 10390 was observed. The upstream sequences of CAMP factor 4 were more variable. The CAMP factor 4 gene for all isolates examined had a GTG start codon, whereas the start codon for all other CAMP factors was ATG (Fig. 5).

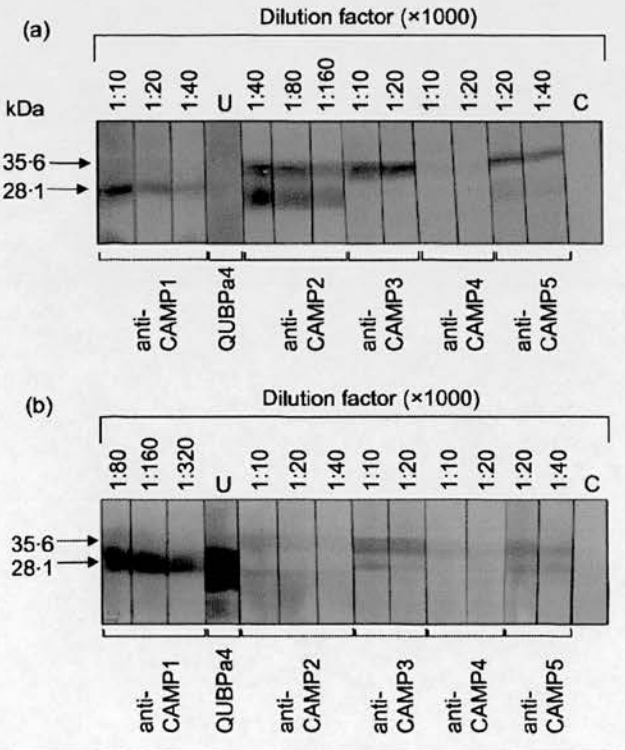

Fig. 3. Immunoblots probed with rabbit anti-CAMP factor protein polyclonal antisera and mouse anti-CAMP factor 1 mAb (QUBPa4). (a) *P. acnes* NCTC 737 (type IA); (b) *P. acnes* SG2 (type II). C, Control with no primary antibody; U, undiluted.

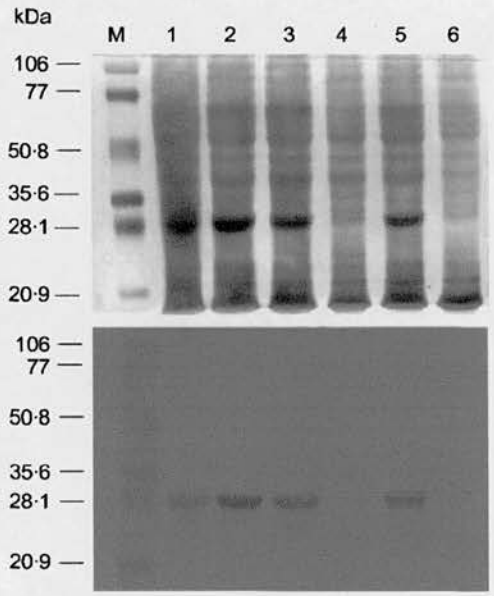

Fig. 4. Analysis of *P. acnes* extracts by SDS-PAGE followed by silver staining (top) or immunoblotting with a mouse anti-CAMP factor 1 mAb (QUBPa4) (bottom). Lanes: 1–3 and 5, type II *P. acnes* isolates JJK1 (lane 1), HMJ3 (2), SG2 (3) and PC3 (5); 4 and 6, type IA *P. acnes* isolates SB1 (4) and AT3 (6). Lanes M, molecular mass markers (Bio-Rad, low range).

CAMP 1	*
NCTC 10390	CGATGAAC**GGAA**CCCACAAATG
NCTC 737	CGATGAA**AGGAA**CCCACAAATG
SG2	CGATGAA**AGGAA**CCCACAAATG
CAMP 2	
Consensus	CAATAAAC**GGAG**AACCTTTATG
CAMP 3	
Consensus	TCCCTCGA**GGAGG**ATTCCCATG
CAMP 4	
NCTC 737	CTGCGATTGT**AG**ATTCATCGTG
KPA171202	CTGCGATT**GGAG**ATTCATCGTG
SG2	CTGCGATT**GGAGG**TTCATCGTG
CAMP 5	
Consensus	CGAAAAAGA**GGAG**AACTCTATG

Fig. 5. Comparison of upstream regions of *P. acnes* CAMP factor genes. Consensus sequence for *P. acnes* NCTC 737 (type IA), KPA171202 (type IB) and SG2 (type II). Base matches representing equivalent DNA sequence of putative core Shine–Dalgarno sequences are illustrated in bold. *, Mismatch in NCTC 10390.

Comparison of CAMP factor 1 expression by *P. acnes* type I and II isolates

Comparison of CAMP factor 1 expression between types IA, IB and II was performed by silver staining of bacterial extracts resolved on SDS-PAGE gels. Confirmation that the abundant protein band observed was CAMP factor 1 was obtained by immunoblotting with our mAb specific for the CAMP factor 1 protein (QUBPa4; Fig. 4). Specificity of the mAb was confirmed by N-terminal sequencing of affinity-purified complete and trypsin-digested protein (data not shown). The staining intensity of the 28 kDa CAMP factor 1 band in extracts from NCTC 737 and NCTC 10390 was compared, as well as 76 type IA, 5 type IB and 31 type II isolates recovered from a variety of sources. The type IA strain NCTC 737 was used as an internal standard on all gels (arbitrary value of 1·0). All 31 type II *P. acnes* isolates, from failed prosthetic hip joints and associated bone and tissue samples, as well as one acne isolate, had a band intensity for CAMP factor 1 that was at least sixfold greater than that for NCTC 737 (mean = 14). The type II strain NCTC 10390 was an exception and lacked an intense band in this region of the gel, consistent with its absence after immunoblotting (not illustrated). The five type IB isolates recovered from dental sources ($n=2$), a failed prosthetic hip joint ($n=1$), a prosthetic hip joint-associated tissue sample ($n=1$) and a skin sample from the hip of a patient undergoing revision arthroplasty ($n=1$), also had at least sixfold greater band intensity for CAMP factor 1 (mean = 18). Type IA isolates recovered from dental sources ($n=6$), failed prosthetic hip joints and associated bone and tissue samples ($n=52$) and patients with acne ($n=18$) had a band intensity in the range of 1–9 (mean = 3).

Immunofluorescence labelling of CAMP factor 1 on whole cells of *P. acnes*

IFM with mAb QUBPa4 indicated that the CAMP factor 1 protein was both cell-associated and secreted (Fig. 6a). Mouse mAbs of the same isotype as QUBPa4, specific for cell-surface components of *P. acnes* I and II, respectively, did not label extracellular material by IFM (Fig. 6b, c). A centrifugation wash in PBS of bacteria harvested from agar plates was sufficient to remove most of the CAMP factor 1 protein (Fig. 6d, e). In addition, we also found that the protein could be obtained from broth culture after 50-fold concentration by ethanol precipitation. *P. acnes* type II strain NCTC 10390, which did not react with QUBPa4 by immunoblotting, was also non-reactive by IFM. IFM revealed no reactivity of QUBPa4 with *P. granulosum*, *P. acidipropionici*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae* (data not shown). Although *A. israelii* and *A. naeslundii* showed no reaction with QUBPa4 by IFM, reactivity was observed after SDS-PAGE and immunoblotting (data not shown).

Reactivity with patient antisera

The sera from acne patients ($n=9$) and patients undergoing primary total hip arthroplasty ($n=10$) and revision

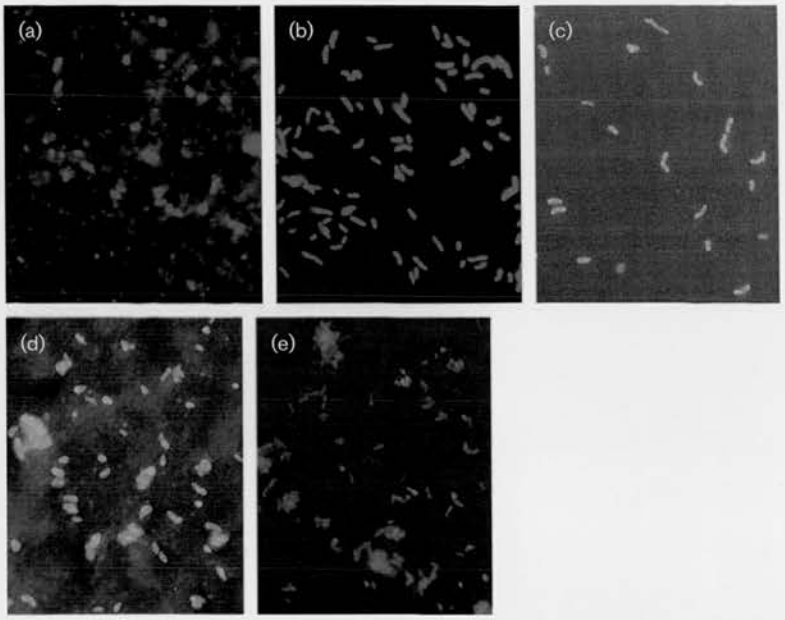

Fig. 6. Immunofluorescence micrographs of *P. acnes* clinical isolates labelled with mouse isotype IgG2b mAbs. (a)–(c) Type II isolate labelled with QUBPa4, specific for CAMP 1 (a), type II isolate labelled with QUBPa2, specific for *P. acnes* type II-associated carbohydrate antigen (b) and *P. acnes* type IA clinical isolate labelled with mouse isotype IgG2b mAb QUBPa5, specific for a protein antigen associated with type IA (c). (d)–(e) Unwashed and washed *P. acnes* type IA clinical isolate labelled with CAMP factor 1-specific QUBPa4: bacterial suspension dried and fixed directly onto slide (d) and bacterial suspension first centrifuged and then resuspended in PBS (e). Images were viewed with fluorescein-detecting filter (a, b) or with combined fluorescein- and Evans Blue-detecting filter (c–e). Microscope magnification, ×1000.

arthroplasty ($n=11$) were examined by immunoblotting at a single dilution of 1:500 for their reactivity against immunoaffinity-purified CAMP factor 1 protein from a type II isolate (SG2). In total, sera from three patients with acne, five patients undergoing primary arthroplasty and four patients undergoing revision arthroplasty were reactive.

DISCUSSION

A total of five individual genes with sequence identity to the co-haemolytic CAMP factor, originally described in *Streptococcus agalactiae* (Christie *et al.*, 1944), were identified in the draft genome sequence of the *P. acnes* type IA strain NCTC 737. Recently, these genes have been independently identified in the genome sequence of the *P. acnes* strain KPA171202 (Bruggemann *et al.*, 2004), which we identified as type IB based on comparison of *recA* and *tly* sequences (McDowell *et al.*, 2005). PCR analysis of a selection of type IA, IB and type II isolates indicated that all five genes are present in each of the different *P. acnes* types. To our knowledge, multiple CAMP homologues have not been described in any other organisms. The conserved nucleotide and amino acid sequence differences observed with the CAMP factors from all three *P. acnes* types provided additional evidence for the phylogenetically distinct nature of the groups and confirmed previous results obtained with *recA* and *tly* genes (McDowell *et al.*, 2005). In contrast, CAMP factor sequences from two serotypes of *Streptococcus agalactiae* (serotype III strain NEM316 and serotype V strain 2603V/R) have been shown to be identical (Glaser *et al.*, 2002). In addition, varying numbers of nucleotide and amino acid differences were observed between the sequences of NCTC 737 (1A) and KPA171202 (1B) for a selection of putative secreted and cell-surface-associated proteins, providing further evidence for their designation as distinct phylogenetic groups within type I (McDowell *et al.*, 2005).

Analysis of CAMP factor protein expression by immunoblotting and silver staining of SDS-PAGE gels revealed an abundance of CAMP factor 1 production by type II and type IB isolates, but not IA organisms. The type II strain NCTC 10390, however, was an exception, as abundant CAMP factor 1 protein was not detected. As the genes for all five CAMP factors are present in all three *P. acnes* groups, observed differences reflected different levels of expression rather than missing genes. One factor amongst many that could influence expression levels is the interaction between a Shine–Dalgarno ribosome-binding site and 16S rRNA. Strong Shine–Dalgarno sequences (e.g. GGAG, GAGG or AGGA) are associated with genes that are predicted to be highly expressed (Karlin & Mrazek, 2000). Interestingly, we identified a base difference in the putative Shine–Dalgarno sequence associated with the CAMP factor 1 gene of NCTC 10390, a type II strain that lacks abundant CAMP factor 1 production and is not reactive with our specific mAb by IFM. Whether or not this reduces the efficiency of interaction with the 16S ribosomal subunit, and therefore the level of expression of the protein, remains to be determined. The observation that NCTC 10390 differs from other type II organisms with respect to CAMP factor 1 production indicates that it is not the most appropriate representative strain of *P. acnes* type II.

Immunoblotting also revealed that type IA isolates express greater quantities of CAMP factor 2 compared with type II and type IB isolates; however, neither this nor the other CAMP factors were produced in quantity by IA strains, as the abundant protein band was not detectable by SDS-PAGE and silver staining. The putative Shine–Dalgarno

sequences of CAMP factors 1 and 2 are conserved for all three phylogenetic groups; therefore, this cannot explain the differences in expression. No striking differences were observed with respect to the expression of CAMP factor proteins 3, 4 and 5, although CAMP factor 4 reacted only weakly in immunoblotting experiments with type IA and II isolates. The putative Shine–Dalgarno sequences upstream of the CAMP factor 4 genes were more varied amongst the three phylogenetic groupings, but comparison with the Shine–Dalgarno sequences of the other CAMP genes did not reveal any clear relationship that could explain the different expression levels. CAMP factor 4 does, however, have a GTG start codon, which is reported to be a weaker translational initiator than ATG (Ringquist *et al.*, 1992).

All of the CAMP factor sequences contain a putative signal sequence cleavage site (Table 2) and molecular mass comparison of the proteins by SDS-PAGE and immunoblotting was in keeping with the loss of this signal sequence. IFM analysis and detection in ethanol-precipitated supernatant from broth culture confirmed that the CAMP factor 1 protein was secreted, although it was also detected on the surface of the *P. acnes* cells. Studies with *Streptococcus agalactiae* similarly detected CAMP factor protein in the external milieu, as well as on the cell surface (Jurgens *et al.*, 1987). None of the CAMP factor homologues from *P. acnes* or *Streptococcus agalactiae* contain a C-terminal Leu-Pro-X-Thr-Gly (LPXTG) motif, although 25 genes encoding other proteins with an LPXTG motif have been described in the genome of *P. acnes* strain KPA171202 (Bruggemann *et al.*, 2004).

Despite our observations of differential expression of the five CAMP factor homologues amongst *P. acnes* types IA, IB and II, all isolates from both groups were positive for the co-haemolytic phenotype. The *P. acnes* co-haemolytic reaction is therefore likely to be mediated by more than one CAMP factor protein. We are currently addressing the relationship between individual CAMP factors and the co-haemolytic phenotype. In our studies, some sequence identity between the *P. acnes* CAMP factor 1 and 3 proteins and *Staphylococcus aureus* protein A was demonstrated within the Fc-binding region, suggesting they may have immunoglobulin-binding activity. Interestingly, the percentage identities were greater than those of the *Streptococcus agalactiae* CAMP factor, which has been shown to bind IgG (Jurgens *et al.*, 1987). It may be that the multiple *P. acnes* CAMP factors have arisen from divergence of a replicated common ancestral gene and now have divergent functions.

In addition to the *P. acnes* isolates studied, *A. israelii* and *A. naeslundii* produced a co-haemolytic reaction whereas *P. granulosum* did not. It would be interesting to determine whether this difference reflects the relative pathogenic potential of these organisms. The positive reaction between purified CAMP factor 1 protein and human sera obtained from patients with acne, as well as those undergoing primary or revision hip arthroplasty, indicates that the protein is expressed by *P. acnes* during human colonization. Whether its production relates to virulence remains to be determined, as the serum samples used were from a single time point and therefore give no indication of rising titre.

In conclusion, we have identified five genes with sequence identity to the co-haemolytic CAMP factor of *Streptococcus agalactiae* in strains of *P. acnes* types IA, IB and II. Differential protein expression of the CAMP factors amongst the various *P. acnes* phylogenetic groupings was observed; in particular, the extracellular and cell-associated CAMP factor 1 protein was produced in striking abundance by type IB and type II isolates. The observation of differential expression of putative virulence determinants amongst the various *P. acnes* types will have important consequences. In particular, it will impact on our interpretation of previously published virulence data that have been based on the study of only one isolate type, such as NCTC 737, which has often been used as a model organism for studies of *P. acnes* virulence (Roszkowski *et al.*, 1980; Webster *et al.*, 1985). More generally, such data, in combination with the recent demonstration that *P. acnes* is genetically heterogeneous, will serve to challenge our current understanding of the virulence and pathogenic potential of clinical isolates of *P. acnes*.

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Evaluation of Nonculture Methods for the Detection of Prosthetic Hip Biofilms

Andrew McDowell, PhD; and Sheila Patrick, PhD

Authors of previous studies have found that culture-based detection of prosthetic hip infections can be improved by adherence to strict anaerobic protocols and mild ultrasonication of retrieved prostheses to remove bacteria growing as an adherent biofilm. Furthermore, direct analyses of sonicate samples by immunofluorescence microscopy (with genus-specific and species-specific monoclonal antibodies or polyclonal antisera) produces significantly greater and more rapid detection rates for infection compared with culture alone. Despite its diagnostic value within a research setting, the practical advantages and limitations of immunofluorescence microscopy for a busy diagnostic laboratory needs consideration. In addition, the method must be evaluated against other nonculture-based techniques that may prove more appropriate. In this review, we describe how immunofluorescence microscopy presents an attractive and reliable method for routine detection of prosthetic hip biofilms compared, primarily, to polymerase chain reaction, although other nonculture methods of diagnosis based on fluorescent in situ hybridization and serologic analysis are considered. We describe how the immunofluorescence microscopy technique is robust, relatively simple and, in contrast to broad-range 16S recombinant-deoxyribonucleic-acid–based polymerase chain reaction, does not require a separate clean-room facility or strict adherence to aseptic techniques. More importantly we highlight how dislodged biofilm, which appears as large aggregates of bacteria, easily can be identified from skin contaminants that occur as single cells or small aggregates of only a few cells.

Total hip replacement surgery is a medical success story and has improved the quality of life considerably for countless people. The majority of patients who receive a prosthetic hip implant have dramatic and immediate relief of pain and regain satisfactory hip function and quality of life.[5] However, a proportion of patients do develop various complications that require further surgery and replacement of the prosthesis (revision arthroplasty).[27] The morbidity and trauma associated with revision arthroplasty is considerable, and in the United Kingdom, incurs large costs to the National Health Service. Although aseptic biomechanical loosening is thought to be the most common underlying cause for prosthetic hip replacement, revision arthroplasties caused by prosthetic hip–associated infections also constitute a substantial burden to the healthcare system and are a disastrous complication for the surgeon.[13] Unfortunately, the rate of infection-related failure for second implants is thought to be even higher than that seen with primary revision surgery.[7] This may be a consequence of the longer operating times involved or unrecognized infection at the time of the revision surgery, which leads to implantation of the second prosthesis without debridement and antimicrobial intervention.[36] In contrast, the incorrect suspicion that a prosthetic hip implant may be infected at the time of revision surgery not only increases the anxiety and stress to the patient, but also results in additional surgery, a prolonged hospital stay, and extra expense. As a consequence, early and accurate diagnosis of prosthetic hip infections would serve to improve the treatment and management of patients considerably.

Although different methods have been used to detect the presence of prosthetic hip infections, no standardized protocol or set of diagnostic guidelines has been universally adopted. Most patients routinely are assessed for the presence of infection before revision surgery using a polyphasic approach that includes clinical history and examination of the patient, measurement of leukocyte count, erythrocyte sedimentation rate and C-reactive protein levels, radiographic and radionuclide imaging, and culture analysis of synovial fluid aspirated from the hip joint (aspiration arthrography).[15,24,36,39] Postoperatively, detection of infection normally is investigated by routine culture analysis of the explanted prosthesis and periprosthetic tissue samples.[36] Authors of histopathology studies also have provided evidence that the presence of inflammatory cells

From the Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Belfast, Northern Ireland.
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Correspondence to: Sheila Patrick, PhD, Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Grosvenor Road, Belfast, BT12 6BN, Northern Ireland. Phone: 44(0)2890 632512; Fax: 44(0)2890 635024; E-mail: s.patrick@qub.ac.uk.
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in periprosthetic tissue biopsies is a valuable marker for the presence of infection. Indeed, researchers who have participated in the Oxford Skeletal Infection Research and Intervention Service (OSIRIS) in the United Kingdom have recommended that patients having revision surgery routinely are evaluated for infection by histologic screening.[2,26] Although the combined use of all these methods usually is sufficient to detect the presence of acute infection that may occur soon after surgery, their diagnostic sensitivity for the detection of chronic low-grade infection may be problematic.

Against this background, we now review nonculture-based methods for the routine detection and identification of prosthetic hip infections. We initially describe how strict anaerobic processing of retrieved prostheses followed by the detection of dislodged prosthetic hip–associated biofilms can improve the diagnosis of prosthetic hip infections. We then highlight the value of nonculture-based methods for the detection of such infections and compare polymerase chain reaction (PCR)-based methods and fluorescent in situ hybridization (FISH) and serologic analysis with immunofluorescence microscopy (IFM). We conclude that IFM currently provides the best diagnostic platform for the detection of prosthetic hip infections and describe how the technique allows dislodged biofilm, which appears as large aggregates of bacteria, to be differentiated from skin contaminants that occur as single cells or small aggregates of only a few cells.

Detection of Prosthetic Hip Joint Biofilms

Authors of previous studies have provided evidence that the incidence of prosthetic hip joint infection is grossly underestimated by various diagnostic methods routinely practiced in clinical laboratories.[37,38] In these studies, infection was identified as a major cause of prosthetic joint failure in 26 of 120 (22%) retrieved prostheses examined.[37,38] Organisms cultured from the prostheses primarily were coagulase-negative staphylococci, mainly Staphylococcus epidermidis and Propionibacterium acnes, which are associated with chronic low-grade infections. When the patient's notes from 18 of these 26 individuals were scrutinized, it was apparent that infection only had been suspected in six cases (33%). Furthermore, in only two cases were bacteria cultured by the routine diagnostic laboratory from preoperative aspirates or periprosthetic tissue removed at the time of surgery. This considerably increased culture-based detection of infection was achieved by immediate placement of the retrieved hip prosthesis in an anaerobic atmosphere, mild ultrasonication (5 min, 50 kHz) of the prosthesis in prereduced quarter-strength Ringer's solution, containing cysteine, to dislodge bacteria growing on the surface as an adherent biofilm, and strict anaerobic processing of the resulting sonicate samples. In addition, direct sampling of sonicate samples by IFM, with a monoclonal antibody (MAb) specific for P. acnes and a polyclonal antiserum specific for Staphylococcus spp., revealed the presence of bacteria in 63% of revised prostheses, providing evidence that culture-based analyses alone may not be the most sensitive diagnostic technique. Molecular detection of infection by broad range 16S recombinant deoxyribonucleic acid (rDNA)-based PCR, using universal primers, seemed to confirm this view because positive PCR results were obtained for 72% of the revised prosthetic hip samples. Although neutrophilic infiltration in periprosthetic tissues has been recommended as a useful nonculture method for the detection of prosthetic hip joint infections, eight of the culture-positive cases (30%) identified in these studies were found to be negative for the presence of neutrophils in prosthetic–hip-associated tissue samples (where available).[37,38] In contrast, lymphocytes and/or macrophages were present, which could be explained, in theory, by the slow release of bacterial components from the prosthetic-hip-associated biofilm. Histopathologic examination of tissue taken from culture-negative patients revealed the presence of high-level neutrophilic infiltration (10 or more neutrophils per high-power field) in eight samples. A further 36 samples with low levels of neutrophils or none at all had high numbers of lymphocytes or macrophages (10 or more lymphocytes or macrophages per high-power field). For a number of these samples, although the tissue pathology score was high, bacteria were present in small numbers by IFM. It therefore remains to be established whether the observed inflammatory response in such samples was driven by wear debris from the prosthesis,[34] bacterial components, or indeed a combination of both. On the basis of these data it has been recommended that current methods routinely used for the detection of prosthetic hip infections be reassessed.[37,38]

Importance of Nonculture Methods for Detection of Prosthetic Hip Joint Biofilms

Because of shorter processing times, nonculture-based methods, such as broad-range 16S rDNA-based PCR and IFM, provide the opportunity for earlier postsurgical detection and identification of prosthetic hip biofilms compared with conventional culture-based approaches, especially those involving anaerobic growth (Fig 1). More importantly, such techniques can facilitate the detection of viable but nonculturable infections in prosthetic hip joints and periprosthetic tissue and bone samples. This may occur because of low numbers of organisms, the use of prophylactic antibiotic therapy, or the fact that bacteria growing within a biofilm community can enter a state of dormancy or quiescence caused by inappropriate growth conditions, which contributes to their increased resistance

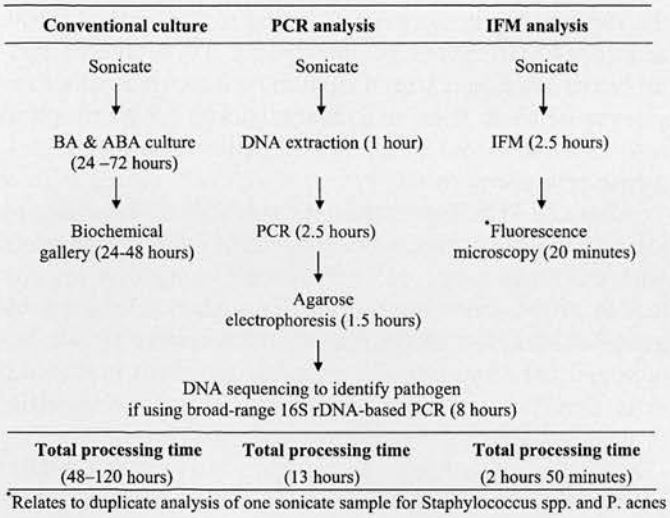


Fig 1. A comparison of conventional culture, PCR, and IFM processing times for the detection and identification of prosthetic hip infections in the routine diagnostic laboratory is shown. Sonicate samples are prepared by mild ultrasonication (5 min, 50 kHz) of the retrieved prosthesis in prereduced ¼-strength Ringer's solution containing cysteine.

to antimicrobials.[11,31] Also, it cannot be discounted that organisms growing as a biofilm may become so highly adapted to their environment that the fastidious growth requirements necessary for their cultivation are not provided within the laboratory setting.[37] Clearly, the contribution that nonculture-based methods make to the detection of prosthetic hip joint infections should not be undervalued. Their capacity to provide faster post-surgical detection of infection, as well as the identification of patients with prosthetic hip infections that traditional approaches may have missed, will facilitate earlier and more aggressive antimicrobial intervention and therefore will educe the possibility that a new infection will be established. This will substantially contribute to the universal goal of improving and refining patient treatment and care.

Comparison of Nonculture-Based Methods for the Detection of Prosthesis Hip Joint Biofilms

Polymerase-Chain-Reaction–Based Protocols

Although nonculture-based methods may prove to be valuable for the detection of prosthetic hip joint biofilms within a research setting, the practical advantages and limitations of such approaches for routine detection of such infections within the context of a busy clinical microbiology laboratory must be considered. Because most modern diagnostic laboratories now have access to thermal cyclers it is only natural that nonculture detection methods based on PCR technology are adopted. Such an approach is even more obvious when one considers that universal or broad-range 16S rDNA-based PCR, using highly conserved oligonucleotide primers, provides an ideal molecular platform for the detection of almost all types of bacterial infection with normally high sensitivity.[10,21,43] However, despite the initial attractiveness that PCR may offer for the detection of prosthetic hip joint infections, a number of potential caveats associated with the use of the technique, especially within a routine laboratory, do exist. Most notably, the exquisite sensitivity that is often associated with PCR detection methods can be a considerable burden practically and economically for the diagnostic laboratory, which must ensure that the positive result obtained for a patient's sample is not caused by coamplification of contaminating DNA.[4,22] Indeed, based only on this, many of laboratories have decided not to adopt broad-range 16S rDNA-based PCR as part of their diagnostic service. Potential DNA contamination can arise from a variety of different sources, including nonsterile sites surrounding the clinical specimen, the specimen vial, surgical or laboratory personnel, aerosols and reagents used for the extraction of DNA and PCR setup, and even unrelated PCR activities in adjacent laboratories.[4,22,32,43] In addition, contamination from pipettes and other equipment, such as PCR cabinets and reaction vials, also can occur. The detection of contamination from nonsterile sites particularly is an important consideration when analyzing retrieved prosthetic hip joints because the organisms frequently responsible for infection, such as Staphylococcus spp, also are part of the normal skin microbiota. To help avoid contamination during broad-range 16S rDNA-based PCR, a wide range of preventative measures need to be taken. These include the use of molecular grade reagents that must be screened for artifactual DNA before use, the wearing of sterile gloves and protective clothing, and dedicated rooms for DNA extraction and PCR setup that contain safety cabinets fitted with ultraviolet lamps (for DNA degradation), pipettes, filtered pipette tips, reaction vials, and other general equipment that must remain on site.[4,22] Elimination of potential contaminants from PCR reagents and equipment can also be achieved by enzymatic and chemical treatments. Furthermore, when carrying out experiments it is imperative that appropriate positive and negative controls (for DNA extraction and PCR setup) are included in each diagnostic run. The use of internal amplification controls also will serve to highlight any false-negative results that may arise from PCR inhibitors within the clinical sample.[43] Although the use of control samples provides some reassurance that contamination of clinical samples has not occurred, it does not completely eliminate such a possibility. For example, we recently identified the presence of artifactual DNA from a β-proteobacterium in batches of sterile irrigation fluid routinely used during

prosthetic hip surgery. Retrospective sequencing studies now also have identified the presence of this DNA in a number of sonicate samples analyzed in our earlier study.[37] As a consequence, the 72% detection rate previously reported for the presence of bacteria in failed prosthetic hip implants by broad-range 16S rDNA-based PCR was an overestimate. The detection of a number of false-positive samples, even when all controls gave expected results, strikingly highlights the potential complications that can arise when using a broad-range 16S rDNA-based PCR approach.

Researchers who conducted previous studies in our laboratory showed that PCR can be used reliably to detect Staphylococcus organisms to concentrations of 10^4 cfu/mL, whereas detection limits for P. acnes reliably do not go below 10^5 cfu/mL.[37] To date, results of ongoing studies with various DNA extraction protocols and broad-range 16S rDNA primer sets have not improved on these detection limits (data not published). Detection limits for the PCR-based diagnosis of gram-positive bacteria can be problematic because of the robust nature of the cell envelope, which is composed of a 3-dimensional matrix of polymers, primarily peptidoglycan and techoic and/or lipoteichoic acids. In addition, P. acnes is a member of the actinomycetales, which are known to have additional cell envelope polymers, making them even more robust than other Gram-positive bacteria. This may therefore explain, at least in part, why it is especially difficult to optimally extract P. acnes genomic DNA. Although this relatively poor detection limit may negate the possibility that low levels of P. acnes contamination from nonsterile sites or surgical or laboratory personnel would be detected, it also does raise the possibility that smaller numbers of the organism that may be present in a chronic infection would be missed. Although the use of a nested or semi-nested PCR method undoubtedly would improve the detection of P. acnes, we are uncomfortable with such an approach for diagnosis because of the greater risk of cross-contamination (from first-round amplification) during routine processing.

Despite the fact that broad-range 16S rDNA-based PCR offers the potential to detect all types of bacterial organisms it does not directly identify the specific nature of the infection without further laboratory investigations. This usually is achieved by DNA sequence analysis of the 16S rDNA amplicon followed by comparison of the sequence to those previously deposited in global sequence databases, such as GenBank at the National Center for Biotechnology Information, and the Ribosomal Database Project.[6,14] However, in addition to increasing the laboratory processing time (Fig 1), this approach will not work when a polymicrobial hip infection is present. In such circumstances, PCR amplicons for each species present in the sample may be generated leading to nonsensical DNA sequences that cannot be interpreted. To overcome this problem, amplicons would have to be separated, either by cloning or some form of polyacrylamide gel electrophoresis, which would delay identification and further increase processing times.[41,42]

Alternate PCR approaches are available to the molecular microbiologist to circumvent the problems associated with the broad-range 16S rDNA-based approach for detection of prosthetic hip biofilm. These include the use of genus or species-specific primers, which serve to reduce, although not eliminate, the potential problems that could arise from contamination. The use of species-specific primers would also negate the necessity for subsequent DNA sequence analysis as the organism could be directly identified from the positive PCR result. Such specific primer sets still can be based on the 16S rDNA locus, although other nonribosomal targets frequently are used with great success.[12,18] These alternate targets are often less well conserved than 16S rDNA sequences affording more opportunity to design specific primers that will differentiate closely related organisms. For example, we recently found that P. acnes serotypes I and II were almost indistinguishable based on 16S rDNA sequences.[20] However, analysis based on the recA housekeeping gene revealed considerably greater serotype-specific polymorphisms that will facilitate the generation of diagnostic primers specific for each type.[20] Another example is the glyceradehyde-3-phosphate dehydrogenase gene, which is a useful tool for the differentiation of most Staphylococcus spp.[45] One obvious disadvantage with the use of genus-specific or species-specific primers would be the possibility that certain pathogens may not be detected from an infected hip prosthesis. To reduce this possibility, multiple primer sets specific for the organisms most likely to be detected would have to be used. Realistically, this would include primers for various Staphylococcus spp., primarily S. aureus and S. epidermidis, and primers for P. acnes. The use of genus-specific primers for Staphylococcus spp. also may be useful and would highlight the presence of other species in addition to S. aureus and S. epidermidis. These individual PCR tests could be combined into a multiplex assay to save time during setup and also reduce the number of samples to process.[12,19] However, optimization of such assays can prove problematic depending on the number of primer sets to be used and their compatibility with respect to annealing temperatures. Also, each primer set must generate different product sizes that are easily distinguished on an agarose gel.

Fluorescent In Situ Hybridization

In addition to PCR, the discriminatory potential of 16S rRNA sequences also could be used for the detection of

prosthetic-hip–associated bacteria by FISH. In this technique, a single oligonucleotide probe (15–30 nucleotides) that has been linked covalently to a fluorescent dye at the 5′ end is hybridized to ribosomes present within the bacterial cell.[1,23,30,40] After stringent washing, the stained cells are detected by fluorescence microscopy or flow cytometry. Fluorescent in situ hybridization not only provides the opportunity to observe and identify microbial cells within clinical samples, but, in combination with confocal laser scanning microscopy, it can be used to reconstruct the spatial arrangement of infecting organisms within their habitat.[40] As with broad-range 16S rDNA-based PCR, universal probes that will react with all bacteria can be used or, alternatively, more specific probes can be designed that discriminate between different bacterial species.[30] The method has been used to study complex microbial communities in the oral cavity, the gastrointestinal tract, and infections of the respiratory tract and pathogenic organisms in blood culture bottles.[23] Although this technique is appealing for the detection of prosthetic hip biofilms because of its potential for direct observation of bacteria, and the relative low cost and ease with which different oligonucleotide probes can be produced, there are a number of practical problems that remain to be overcome. In particular, autofluorescence of the organisms themselves, or material surrounding the bacteria, can decrease the signal-to-noise ratio and hinder detection of specific fluorescent signals.[23] Also, the complex nature of the cell envelope in gram-positive bacteria, especially those exhibiting long or medium chain mycolic acids, and the large extracellular polysaccharide capsules present on some gram-negative organisms, can create problems relating to insufficient penetration of the oligonucleotide probe into the bacterial cell.[23,30,40] The recent introduction of peptide nucleic acid probes, which have an uncharged polyamide backbone, may help to reduce this problem because they have the capacity to diffuse through hydrophobic cell walls.[25,28] However, the expensive nature of such probes and problems associated with their specificity currently are hindering their widespread application. Problems also can arise because of the accessibility of the oligonucleotide probe to target sites on the 16S rRNA molecule. This occurs because of loop and hairpin formations in the higher-order structure of the ribosome and protein-rRNA interactions. The use of unlabelled helper probes, which bind adjacent to the probe target site and are thought to open up higher order structures that hinder binding, can enhance detection.[9] Other issues that can arise when using FISH include the relationship between ribosome number and fluorescent signal. The content of rRNA can vary considerably between cells of a given strain because of their physiologic state, which is dependent on their phase of growth.[30] This particularly is pertinent to slow-growing or dormant biofilm bacteria in which ribosome numbers likely are to be low. As a consequence, low-signal intensity or a false-negative result may occur. Attempts to address this issue have focused on increasing cellular ribosome content by incubating samples with a cocktail of substrate and antibiotics that cause cell activation and rRNA synthesis without cell division.[16] Although this can lead to an increase in signal intensity, the caveat with such an approach is the selectivity of the substrates and antibiotics. Consequently, although FISH remains an extremely useful research tool, its application to routine diagnosis in the short term may be troublesome.

Serologic-Based Analysis

Nonculture-based serological detection of gram-positive infections on prosthetic hip implants by enzyme-linked immunosorbent assay (ELISA) also has been described.[29] This method measures the serum immunoglobulin G response to a short-chain length form of lipoteichoic acid that is present in the cell membrane and extracellular material of Staphylococcus spp. The technique also has been applied to the detection of gram-positive infections associated with intravenous catheters and sciatica.[8,33] The sensitivity (93.3%) and specificity (96.9%) of the ELISA has provided evidence that it could be useful in the detection of occult prosthetic hip infections. Furthermore, because the method is quick and relatively inexpensive, it may be easily adapted into the diagnostic laboratory. However, authors of the original ELISA study only examined 15 patients with proven prosthetic hip infections; therefore evaluation of this technique with a much greater number of patients would be required to fully assess its diagnostic value.[29] Furthermore, as the technique is not specific for prosthetic hip infections; patients with other bacterial infections at the time of analysis or those with a recent history of infection may not be suitable for testing. A further problem with this method is the presence of antibodies to the staphylococcal antigen within the normal population. As a consequence, basal IgG titer levels need to be evaluated for the population being studied. The determination of a suitable titer cut-off point is somewhat subjective, and the possibility also remains that titers may overlap between the patient and control groups.

Immunofluorescence Microscopy

An alternative nonculture detection method for prosthetic hip biofilms based on IFM is being developed (Fig 2).[37] In this approach, adherent biofilms dislodged from the prosthesis by mild ultrasonication are concentrated and directly fixed to the wells of a glass slide before incubation with a MAb or polyclonal antisera specific for the organism(s)

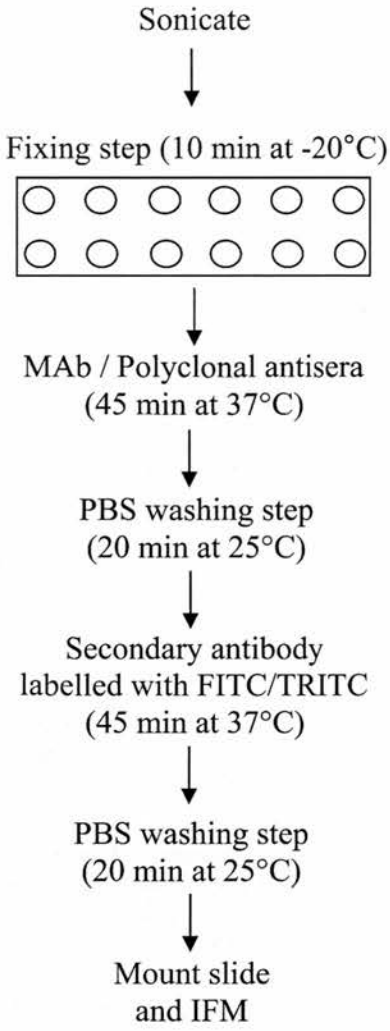
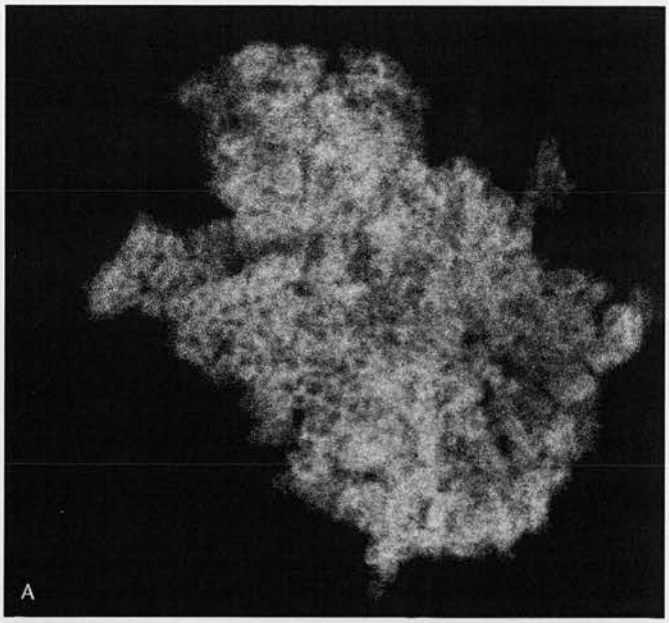


Fig 2. An IFM-based protocol for the detection and identification of prosthetic hip infections is shown. Sonicate samples are prepared by mild ultrasonication (5 min, 50 kHz) of the retrieved prosthesis in prereduced ¼-strength Ringer's solution containing cysteine.

under investigation. After incubation with a secondary antibody conjugated to a fluorogenic dye, such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), bacteria present in the samples can be detected by fluorescence microscopy (indirect labeling). With specific regard to the detection of prosthetic hip biofilms, we think this approach has a number of key advantages over a PCR-based method and, consequently, may be more suitable for routine diagnostic laboratories. In particular, the current IFM method has a considerably shorter processing time compared with PCR, which means the clinician can be informed of any underlying infection within an earlier timeframe (Fig 1).[37] Also, the IFM-based method provides the opportunity to distinguish bacterial contamination from a real infection, which is one of the critical advantages of the technique. In conventional PCR, the basis of a positive result is the detection of a band of the correct size on an agarose gel. However, even when all positive and negative control samples are correct, there still remains the possibility that the clinical specimen itself could have been contaminated, which observation of a DNA band will not highlight. In contrast, with IFM the bacterial cells can be seen directly (with coryneform or cocci morphology), which facilitates identification of contaminating organisms from those that have been growing as an adherent biofilm on the prosthesis. On the basis of control experiments conducted in our laboratory, as well as our experience processing large numbers of retrieved hip prostheses, organisms that we normally would classify as contaminants are observed infrequently within the well of the slide and occur either as single cells or a small aggregates composed of only a few cells.[37] In contrast, dislodged biofilm can be easily identified as large aggregates of bacterial cells consisting of several layers (Fig 3). These bacterial aggregates, which vary in depth from 3.5 to 4.0 μm, are only ever seen after sonication, but never after a simple diluent wash of the prosthesis.[37] Because of the differences in bacterial morphology for coryneform and cocci species, the combined use of antibodies specific for P. acnes and Staphylococcus spp. also enables any dislodged biofilm containing both types of organisms to be observed (Fig 4). Although the use of specific MAbs or polyclonal antisera enables the nature of the infecting organism(s) to be directly identified, it does create the possibility that other organisms unreactive with the antibodies would not be identified. Although this is a potential limitation of the technique, the introduction of additional MAbs or antisera raised to other putative pathogens would improve this situation. To illustrate this point, we have improved our detection of prosthetic hip infections by generating MAbs specific for the two serotypes of P. acnes (Types I and II).[20]

We have found IFM-based detection of prosthetic hip biofilms to be a very robust technique that does not require much expertise or training to do. Apart from the initial cost of a fluorescence microscope, the method is relatively inexpensive and does not require the scrupulous adherence to aseptic technique that is required to eliminate DNA contamination when using broad-range 16S rDNA-based PCR. Indeed, the technique can be conducted comfortably on the laboratory bench once the sonicate sample has been prepared and fixed to the microscope slide. Furthermore, our IFM method does not suffer from the various problems that can arise when using a FISH-based approach, such as insufficient penetration of the oligonucleotide probe into the bacterial cell or accessibility of the oligonucleotide probe to target sites on the 16S rRNA molecule. As a



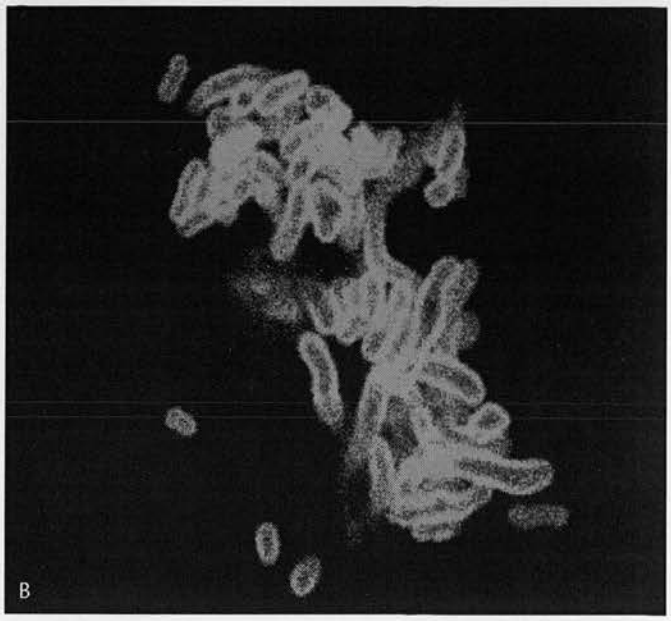


Fig 3. A confocal laser-scanning micrograph of bacterial biofilms removed from retrieved prostheses by ultrasonication is shown. Biofilms were labeled with either (A) anti-Staphylococcus spp. antiserum (1/400) or (B) undiluted anti-P. acnes MAb hybridoma culture supernatant (QUBPa3) before incubation with a secondary antibody conjugated to a fluorescent dye (FITC).

consequence, the method represents an attractive nonculture-based method that could be implemented, at least in the short term, for the detection of prosthetic-hip–associated infections. The IFM technique also could be applied to other medical devices used in orthopedics, such as intramedullary nails and devices used in other areas of medicine, such as prosthetic heart valves.

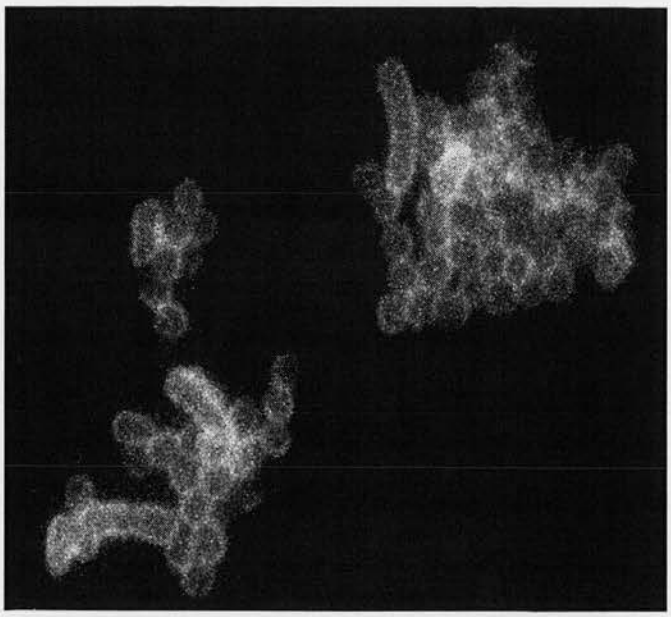


Fig 4. A confocal laser-scanning micrograph of a bacterial biofilm dislodged from a retrieved prosthesis by ultrasonication is shown. The biofilm was labeled with an anti-Staphylococcus spp. antiserum (1/400) and an undiluted anti-P. acnes MAb hybridoma culture supernatant (QUBPa3) before incubation with a secondary antibody conjugated to a fluorescent dye (FITC). Note cocci and coryneform morphology of Staphylococcus spp. and P. acnes respectively, which allows visual differentiation of the organisms.

DISCUSSION

Although IFM seems to be the most appropriate nonculture method currently available for the reliable detection of prosthetic hip biofilms, it is important to recognize that continuing technological advances likely are to develop improved or alternate nonculture approaches to those used currently. In particular, real-time PCR offers a notable improvement on conventional PCR methods because of its capacity for greatly reduced assay times and postamplification analysis.[17] This particularly could be advantageous for the detection of prosthetic hip infections. The method also offers increased specificity and sensitivity, as well as the capacity to identify background amplification products based on quantification and melting curve analysis. Currently, the main limitation with this approach is the relatively expensive nature of the technology, which prevents it from being a routine feature for many diagnostic laboratories. Other developments in the molecular field that are likely to directly impact the detection of prosthetic hip

infections include the use of DNA microarray or DNA chip technology.[3,44] For example, the use of diagnostic microarrays containing species-specific 16S rDNA oligonucleotide sequences (20–25 nucleotides) from a wide range of pathogens would allow the simultaneous detection and identification of organisms in one experiment. This greatly would reduce the time taken for diagnosis compared to conventional culture-based methods. Currently, the use of DNA-microarray technology is not financially viable for most diagnostic laboratories. Furthermore, for an 'average' laboratory, setting up an 'in-house' microarray does require a great level of expertise ranging from the construction of an arraying robot to the design of multiple species-specific 16S rDNA oligonucleotides and computer-based analysis and interpretation of the output data.[35] Despite these current economic and practical limitations, microarrays likely are to become routine diagnostic tools within every laboratory in the future as methodologies improve and equipment costs decrease.

In the more immediate future, now that we have established and validated the IFM-based approach for the detection of prosthetic hip biofilms, we hope to streamline the technique so that the time from receipt of the retrieved prosthesis to identification of infection can be considerably reduced. Currently, we are optimizing our washing procedures for the glass slides and eliminating the secondary antibody incubation step by directly labeling our MAb and polyclonal antisera with FITC and TRITC, respectively. This will reduce our current processing time for the procedure (Fig 1), and also further simplify the technique. Ultimately, the goal is to develop a rapid IFM-based method to enable detection of infection before the end of surgery, guiding the clinician on the most appropriate course of treatment such as a delayed two-stage revision procedure.[46]

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Propionibacterium acnes Types I and II Represent Phylogenetically Distinct Groups

Andrew McDowell,[1] Susanna Valanne,[1] Gordon Ramage,[1] Michael M. Tunney,[1] Josephine V. Glenn,[1] Gregory C. McLorinan,[1] Ajay Bhatia,[2] Jean-Francois Maisonneuve,[2] Michael Lodes,[2] David H. Persing,[2] and Sheila Patrick[1]*

Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Belfast, United Kingdom,[1] and Corixa Corporation, Infectious Disease Research Institute, Seattle, Washington[2]

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Although two phenotypes of the opportunistic pathogen *Propionibacterium acnes* (types I and II) have been described, epidemiological investigations of their roles in different infections have not been widely reported. Using immunofluorescence microscopy with monoclonal antibodies (MAbs) QUBPa1 and QUBPa2, specific for types I and II, respectively, we investigated the prevalences of the two types among 132 *P. acnes* isolates. Analysis of isolates from failed prosthetic hip implants ($n = 40$) revealed approximately equal numbers of type I and II organisms. Isolates from failed prosthetic hip-associated bone ($n = 6$) and tissue ($n = 38$) samples, as well as isolates from acne ($n = 22$), dental infections ($n = 8$), and skin removed during surgical incision ($n = 18$) were predominately of type I. A total of 11 (8%) isolates showed atypical MAb labeling and could not be conclusively identified. Phylogenetic analysis of *P. acnes* by nucleotide sequencing revealed the 16S rRNA gene to be highly conserved between types I and II. In contrast, sequence analysis of *recA* and a putative hemolysin gene (*tly*) revealed significantly greater type-specific polymorphisms that corresponded to phylogenetically distinct cluster groups. All 11 isolates with atypical MAb labeling were identified as type I by sequencing. Within the *recA* and *tly* phylogenetic trees, nine of these isolates formed a cluster distinct from other type I organisms, suggesting a further phylogenetic subdivision within type I. Our study therefore demonstrates that the phenotypic differences between *P. acnes* types I and II reflect deeper differences in their phylogeny. Furthermore, nucleotide sequencing provides an accurate method for identifying the type status of *P. acnes* isolates.

Propionibacterium acnes is an opportunistic pathogen implicated in late-stage prosthetic joint infections, acne vulgaris, endocarditis, endophthalmitis, osteomyelitis, and shunt-associated central nervous system infections (2, 5, 7, 33). Currently, routine diagnostic practices may underestimate the clinical importance of this anaerobic organism due to inefficient detection and isolation procedures, along with the traditionally held view that, due to its low virulence, its presence in clinical samples reflects contamination. While the opportunistic pathogenic potential of coagulase-negative staphylococci (CoNS), such as *Staphylococcus epidermidis*, is well recognized, the importance of *P. acnes* may still be overlooked (13), despite the fact that it produces more kinds of putative virulence determinants than CoNS (5, 38). This fact is illustrated by recent studies in which *P. acnes* was recovered as frequently as CoNS from the prosthetic hips of patients undergoing revision arthroplasty (33, 34).

As a member of the resident human microbiota, *P. acnes* is found predominantly in the sebaceous gland-rich areas of the skin in adults (5, 25). It has, however, also been isolated from the conjunctiva, the mouth, and the large intestine (7). It accounts for approximately half of the total skin microbiota (31), with an estimated density of 10^2 to 10^5 or 10^6 organisms per cm^2 (18, 25). Studies by Johnson and Cummins (14) first revealed two distinct phenotypes of *P. acnes*, known as types I and II, that could be distinguished based on serological agglutination tests and cell wall sugar analysis. The GC contents and DNA homologies of types I and II were found to be similar to each other but significantly different from those of *Propionibacterium granulosum* and *Propionibacterium avidum* (14). Other methods for differentiating the two serotypes, based on bacteriophage (39) and fermentation typing (10, 16), as well as immunofluorescence with polyclonal antisera (11), have also been described. To date, serological methods have proved to be more specific and/or practical than other approaches for the differentiation of types I and II.

The roles that *P. acnes* types I and II play in various clinical infections, and the potential differences in their production of putative virulence factors, have not been widely examined. Furthermore, although serological and biochemical analyses of *P. acnes* have revealed two distinct phenotypic groups, no phylogenetic study of these biovars has been performed. Improvements in the methods used to differentiate *P. acnes* types I and II, and a better understanding of their phylogeny, will greatly aid epidemiological and virulence studies of this opportunistic pathogen and facilitate the investigation of potentially pathogenic strains associated with specific clinical conditions.

In this study, we have improved antibody-based identification of *P. acnes* types I and II by generating type-specific

* Corresponding author. Mailing address: Department of Microbiology and Immunobiology, School of Medicine, Queen's University, Grosvenor Road, Belfast, BT12 6BN, United Kingdom. Phone: 44 (0) 2890 632512. Fax: 44 (0) 2890 635024. E-mail: s.patrick@qub.ac.uk.

monoclonal antibodies (MAbs) that enable differentiation by immunofluorescence microscopy (IFM). We have also conducted a phylogenetic comparison of type I and II isolates recovered from various sources. We now report that the phenotypic differences observed by antibody labeling reflect deeper differences at the gene level, and we propose that types I and II represent phylogenetically distinct cluster groups.

MATERIALS AND METHODS

Bacterial strains. The following reference strains were from the National Collection of Type Cultures (NCTC; Colindale, England) or the American Type Culture Collection (ATCC; Manassas, Va.): *P. acnes* (NCTC 737; NCTC 10390), *Propionibacterium granulosum* (NCTC 10387), *Staphylococcus aureus* (NCTC 10788; ATCC 25923), *Staphylococcus epidermidis* (NCTC 11047), *Staphylococcus haemolyticus* (NCTC 11042), *Staphylococcus hominis* (NCTC 11320), *Staphylococcus intermedius* (NCTC 11048), *Staphylococcus lugdunensis* (NCTC 12217), *Staphylococcus xylosus* (NCTC 11043), *Streptococcus equi* (NCTC 3767), *Streptococcus lactis* (NCTC 66430), *Streptococcus mitis* (NCTC 3767), *Streptococcus mutans* (NCTC 10449), *Streptococcus pyogenes* (NCTC 11200), *Streptococcus salivarius* (NCTC 163034), *Streptococcus sanguinis* (NCTC 3753), *Peptostreptococcus magnus* (NCTC 11804), and *Bacteroides fragilis* (NCTC 9343). A total of 132 *P. acnes* isolates were examined. Sixty-five isolates were recovered from failed prosthetic hip joints and associated bone and tissue samples, as detailed previously (33, 34). A total of 18 skin isolates were obtained from spinal operation incision sites after homogenization of skin samples as previously described (33, 34). In addition, a further 19 *P. acnes* isolates from tissue samples associated with failed prosthetic hips, as well as isolates recovered from patients with acne ($n = 22$) and dental ($n = 8$) infections, were kind gifts. Isolates of *Corynebacterium diphtheriae*, *Corynebacterium hofmannii*, *Corynebacterium xerosis*, and *Propionibacterium avidum* were provided by the culture collection of the Department of Microbiology and Immunobiology, Queen's University, Belfast, United Kingdom.

Bacterial culture. All anaerobic strains were grown on anaerobic blood agar (ABA) (CM0972; Oxoid Ltd., Basingstoke, England). Cultures were incubated at 37°C in an anaerobic cabinet (MACS MG 1000; Don Whitley Scientific, Shipley, England) under an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. Aerobic coryneform strains were grown aerobically at 37°C on blood agar (BA). All *Staphylococcus* and *Streptococcus* strains were also grown at 37°C on BA. Isolates of *P. acnes* were identified by using the API 20A multitest identification system (BioMérieux, Basingstoke, England) in accordance with the manufacturer's instructions.

Fermentation tests. Fermentation reactions of *P. acnes* were studied on modified protease peptone yeast agar plates containing 40 mg of bromocresol purple indicator (BDH, Poole, England)/liter and 1% (wt/vol) sorbitol, erythritol, or ribose (Sigma-Aldrich Company Ltd., Poole, England). Organisms were grown anaerobically, as described above, and a positive fermentation reaction was noted if the agar plates turned yellow due to acid production. Fermentation biotypes (biotypes 1 to 5) based on all three substrates were determined for culture collection reference strains NCTC 737 and NCTC 10390; strains AT1, DW1, and ED2, used for MAb production; and strains with atypical MAb labeling (10, 16). For all other isolates, only sorbitol fermentation was routinely investigated for type I organisms.

Production of MAbs. MAbs were generated by using the protocol described previously (33). Four BALB/c mice were immunized with killed whole cells (10^8 CFU/ml) of the *P. acnes* strain AT1 (type I; biotype 3), while a further four BALB/c mice were immunized with a combination of killed whole cells of *P. acnes* strains DW1 and ED2 (type II; biotype 2). Hybridoma cell lines producing *P. acnes*-specific MAbs were cloned by limiting dilution (9).

IFM. A modification of the IFM procedure described by Patrick et al. (27) was performed on pure cultures. Briefly, bacterial cultures were grown for 18 h on ABA or BA, and a suspension of 10^8 CFU/ml in 0.01 M phosphate-buffered saline (PBS, consisting of 0.15 M NaCl, 0.0075 M Na_2HPO_4, and 0.0025 M $NaH_2PO_4 \cdot 2H_2O$ [pH 7.4]) was prepared. Samples (10 μl) were then applied to multiwell slides, air dried, and fixed in 100% methanol for 10 min at −20°C. Undiluted human AB serum (30 μl) (Sigma-Aldrich Ltd.) was added as a blocking agent, and the slides were incubated at 37°C for 45 min. The slides were then washed in 0.01 M PBS containing 0.5% (vol/vol) AB serum for 20 min. The appropriate undiluted MAb-containing supernatant (30 μl) was added to each well of the slides and incubated for 45 min at 37°C. After a wash, as before, wells were incubated with a 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma-Aldrich Ltd.) in a 0.1% Evans blue (Merck Sharp & Dome, Hoddesdon, England) counterstain (30 μl) for 45 min at 37°C. Slides were then washed and mounted in glycerol-PBS containing an antiphotobleaching agent (Citifluor; Agar Scientific Ltd., Stansted, England), and examined by using a Leitz Dialux 20 fluorescence microscope. A selection of slides was also examined by confocal laser scanning microscopy.

Immunogold labeling for electron microscopy. *P. acnes* cultures were suspended in distilled water, and several drops were applied to a glow-discharged Formvar carbon-coated copper grid (400 mesh). Grids were air dried and immunogold labeled as described previously (23). The samples were then incubated with PBS containing 1% (wt/vol) bovine serum albumin (Sigma-Aldrich Ltd.) for 10 min, followed by an undiluted MAb-containing supernatant for 90 min at room temperature. The grids were washed in 20 mM Tris-HCl (pH 8.2) containing 0.9 M NaCl and 0.1% (wt/vol) bovine serum albumin before incubation with goat anti-mouse immunoglobulin G (IgG) conjugated with 15-nm-diameter gold particles (Amersham Biosciences, Little Chalfont, England) for 90 min. After a wash in distilled water, bacteria were negatively stained with ammonium molybdate (2% [wt/vol]) and examined with a transmission electron microscope (Philips CM10).

Preparation of bacterial whole-cell lysates and cell wall extracts. Whole cells (25 mg [wet weight]/ml) were suspended in distilled water and disrupted by ultrasound (Soniprep 150; amplitude, 26 μm) for 5 min at 4°C. Intact lyophilized bacterial cell walls were obtained by using the method of Hancock and Poxton (8). Sterile distilled water was added to the lyophilized cell walls (10 mg/ml) prior to ultrasonication as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Sonicates of whole cells and purified cell wall extracts were analyzed on 9.0% (wt/vol) discontinuous polyacrylamide gels according to the method of Laemmli (17). Separated components were then transferred to a nitrocellulose membrane (Protran; pore size, 0.45 μm; Schleicher and Schuell, Dassel, Germany) by using a Mini TransBlot apparatus (Bio-Rad Laboratories Ltd., Hemel Hampsted, England) according to the manufacturer's instructions. Immunoblotting was carried out as previously described with minor modifications (26). In brief, the nitrocellulose membrane was blocked with 0.01 M PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween) and 0.5% (wt/vol) nonfat milk powder (Marvel; Premier Brands, Spalding, England). After a wash with PBS-Tween, the nitrocellulose membrane was incubated in an undiluted MAb-containing supernatant. The nitrocellulose membrane was then washed in PBS-Tween before incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Sigma-Aldrich Ltd.). Bound MAbs were detected by using an alkaline phosphatase conjugate substrate kit containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad Laboratories Ltd.). Where required, and before the addition of the MAb-containing supernatant, nitrocellulose strips containing electroblotted proteins were preincubated for 1 h at 37°C with different concentrations of proteinase K (Sigma-Aldrich Ltd.) in 20 mM Tris-HCl buffer (pH 8.0) containing 2 mM $CaCl_2$ or with different concentrations of sodium-*meta*-periodate (Sigma-Aldrich Ltd.) in citrate buffer (pH 7.5).

PCR analysis. Bacterial genomic DNA was prepared by boiling a suspension (0.5 ml) of freshly cultured cells in sterile PCR-grade water (LAL grade; Cambrex Bioscience, Wokingham, England) for 10 min. The suspension was then cooled before centrifugation at 15,000 × *g* for 2 min. The resulting supernatant containing genomic DNA was stored at −20°C prior to analysis. PCR amplifications were carried out using a Gene Cycler (PT-225; MJ Research, Inc., Waltham, Mass.). Samples contained 1× PCR buffer, 200 μM each deoxynucleoside triphosphate (Amersham Biosciences), 200 μM each primer (Invitrogen Life Technologies, Paisley, Scotland), 1.5 mM $MgCl_2$, 1.25 U of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies), and 2.5 μl of bacterial lysate in a total volume of 25 μl. The 16S rRNA gene of *P. acnes* (1,484 bp; positions 3 to 1486 of GenBank sequence AB042288) was amplified by using the published universal primers UFPL (5′-AGTTTGATCCTGGCTCAG-3′) and URPL (5′-GGTTACCTTGTTACGACTT-3′) (20). The *P. acnes recA* and *tly* genes were amplified by using primers directed to downstream and upstream flanking sequences of each open reading frame, thus facilitating accurate sequence determination of the 5′ and 3′ ends of each open reading frame. The *recA* primers PAR-1 (−96 to −75) (5′-AGCTCGGTGGGGTTCTCTCATC-3′) and PAR-2 (+1105 to +1083) (5′-GCTTCCTCATACCACTGGTCATC-3′) generated a 1,201-bp amplicon, while the *tly* primers PAT-1 (−85 to −65) (5′-CAGGACG TGATGGCAATGCGA-3′) and PAT-2 (+824 to +803) (5′-TCGTTCACAAG ACCACAGTAGC-3′) generated a 909-bp amplicon. Samples were initially heated at 95°C for 3 min, followed by 35 cycles consisting of 1 min at 95°C, 30 s at 55°C, and 1.5 min at 72°C. The PCR was completed with a final extension step at 72°C for 10 min. A negative control (water) was included in all experiments. All PCR products were analyzed by electrophoresis on 1% (wt/vol) agarose gels

(Invitrogen Life Technologies) containing 1× Tris-acetate-EDTA buffer. Molecular size markers (1-Kb Plus DNA ladder; Invitrogen Life Technologies) were run in parallel on all gels. Resolved DNA products were stained with ethidium bromide and viewed under UV light.

Nucleotide sequence analysis. PCR products were purified by using a QIAquick PCR purification kit (QIAGEN, Crawley, England). Sequencing reactions were performed using ABI PRISM Ready Reaction Terminator cycle sequencing kits (Perkin-Elmer Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Samples were analyzed on an ABI PRISM 3100 DNA sequencer (Perkin-Elmer Applied Biosystems). Raw sequences from both DNA strands were obtained by using the appropriate forward and reverse primers. Internal sequencing primers were also used to facilitate determination of the larger 16S rRNA gene sequence.

Phylogenetic analysis. The phylogenetic relationships of *recA* and *tly* genes were determined by using the Data Analysis in Molecular Biology and Evolution (DAMBE) software (http://web.hku.hk/~xxia/software/software.htm). Multiple sequence alignments were performed by using the CLUSTAL W algorithm (32) and were exported into the DAMBE program. Phylogenetic trees were constructed by the maximum-parsimony method and the neighbor-joining method using the Jukes-Cantor-based algorithm. The sequence input order was randomized, and bootstrapping resampling statistics were performed using 100 data sets for each analysis. Analysis was performed on a selection of isolates chosen to represent different nucleotide sequences.

Nucleotide sequence accession numbers. Table 1 summarizes the *P. acnes* type I and II isolates which were examined for 16S rRNA, *recA*, and *tly* gene sequences. Nucleotide sequences were submitted to GenBank, and each was assigned an accession number as shown in Table 1.

RESULTS

MAb reactivity. Two MAbs (QUBPa1 and QUBPa2) were selected on the basis of their reactivities with *P. acnes* reference strains whose type status was inferred from sugar fermentation reactions. Immunoreactivity was consistent upon repeated testing and was independent of whether the bacterial cells were prepared from plate or broth culture.

QUBPa1 reacted with the cell surface antigens of the *P. acnes* type I reference strain NCTC 737 (biotype 3) (Fig. 1A). IFM and immunoelectron microscopy revealed that the surface antigen localized at the apices and septa of a variable proportion of *P. acnes* cells (Fig. 1A and 2). Within a population, at least 95% of the bacterial cells were labeled with the MAb. QUBPa2 reacted with a cell surface antigen of the *P. acnes* type II reference strain NCTC 10390 (biotype 2) (Fig. 1B). Complete labeling of the bacterial surface by QUBPa2 was observed upon IFM (Fig. 1B). All cells within the population were labeled. QUBPa1 and QUBPa2 showed no reaction with NCTC 10390 and NCTC 737, respectively, as detected by conventional fluorescence microscopy or confocal laser scanning microscopy, even with a 10-fold increase in green channel laser power (data not shown).

Both MAbs were nonreactive with other cutaneous *Propionibacterium* spp., with the coryneform bacteria *C. diphtheriae*, *C. hofmannii*, and *C. xerosis*, and with *B. fragilis*, *Peptostreptococcus magnus*, and all *Staphylococcus* and *Streptococcus* strains, as detailed in Materials and Methods.

Antigen characterization. The antigens interacting with QUBPa1 and QUBPa2 were characterized by SDS-PAGE and immunoblotting on sonicate samples from whole cells and purified cell wall extracts. With QUBPa1, two bands of 61 and 68 kDa were observed for whole-cell sonicates of the *P. acnes* reference strain NCTC 737 (type I) (Fig. 3A). Identical results were also obtained with *P. acnes* type I isolates (data not shown). When cell wall sonicates of NCTC 737 were analyzed, only one band of 68 kDa was detected (Fig. 3A). With QUBPa2, a diffuse band(s) or smear ranging from 30 to 40 kDa and a discrete band at 21 kDa were observed for whole-cell sonicates of the *P. acnes* reference strain NCTC 10390 (type II) (Fig. 3B). Similar results were obtained with *P. acnes* type II isolates (data not shown). Analysis of cell wall sonicates of NCTC 10390 revealed a similar banding pattern (Fig. 3B). No reactivity of QUBPa1 or QUBPa2 with whole-cell preparations of *P. acnes* NCTC 10390 or NCTC 737, respectively, was observed (Fig. 3).

To characterize the antigens further, immunoblots of whole-cell sonicates from NCTC 737 and NCTC 10390 were treated with proteinase K or sodium-*meta*-periodate prior to incubation with the MAbs. With immunoblots of NCTC 737, the type I antigen reactivity with QUBPa1 was sensitive to a proteinase K concentration of 7.8×10^{-4} mg/ml (Fig. 4A). Proteinase K treatment of whole cells prior to IFM analysis also revealed a similar diminution in QUBPa1 reactivity. Treatment of blots with sodium-*meta*-periodate had no effect on reactivity (data not shown). With NCTC 10390, type II antigen reactivity with QUBPa2 was sensitive to a sodium-*meta*-periodate concentration of 5×10^{-2} M (Fig. 4B). Treatment of whole cells with sodium-*meta*-periodate prior to IFM analysis produced a similar reduction in QUBPa2 reactivity. Treatment of blots with proteinase K had no effect on QUBPa2 reactivity (data not shown).

IFM analysis of *P. acnes* isolates. IFM analysis of 132 isolates of *P. acnes* with QUBPa1 and QUBPa2 identified 87 (66%) as type I and 34 (26%) as type II (Table 2). Approximately equal numbers of type I and type II isolates were recovered directly from failed hip prostheses (Table 2). Isolates from associated bone and tissue samples were predominately of type I, as were the majority of isolates recovered from patients with acne or dental (periodontitis, pericoronitis, and endodontic) infections and isolates recovered from skin removed at the time of surgical incision (Table 2). A total of 11 isolates (8%) gave atypical labeling with our MAbs (Table 1). Nine isolates (CK17, JMK9, LED2, MMG9, RM9, TON9, W1392, W1998, WMK9) displayed no reaction with MAb QUBPa1 but were identified as type I by virtue of their capacity to ferment sorbitol, a characteristic of some type I, but no type II, organisms. These isolates did, however, react with QUBPa2, but with significantly reduced fluorescence intensity and with an uncharacteristic reduction in the proportion of the bacterial population labeled with the MAb. This group of *P. acnes* organisms was recovered from failed prosthetic hip implants and associated tissue, patients with acne and dental infections, and skin wounds. Additional analysis with erythritol and ribose sugars revealed that all organisms within this atypical grouping shared the same fermentation biotype (biotype 1). A further two isolates (PV66 and PV93) reacted with both QUBPa1 and QUBPa2. Isolate PV66 exhibited a weak reaction with QUBPa1 but a strong reaction with QUBPa2. In contrast, PV93 was strongly positive with QUBPa1 but also displayed weak reactivity with QUBPa2. Neither of these isolates fermented sorbitol.

The remaining 92% of *P. acnes* isolates ($n = 121$) reacted with either QUBPa1 or QUBPa2, and no cross-reaction between the two MAbs was observed. The visual pattern of the MAb labeling (apex and septum or complete cell surface labeling [Fig. 1]), as well as the proportion of cells labeled, was

TABLE 1. Comparison of *P. acnes* isolates by sorbitol fermentation, MAb labeling, and nucleotide sequence analyses

P. acnes isolate and source	Sorbitol fermentation	Type by MAb labeling[a]	16S rRNA accession no.	*recA* accession no.	Type by *recA* sequencing	*tly* accession no.	Type by *tly* sequencing[e]
Reference collection							
NCTC 737	+	I	AB042288	AY642055	I	AY527219	I
NCTC 10390	−	II	AY642044	AY642061	II	AY644409	II
Acne							
P6	+	I		AY642071	I		ND
P9	+	I		AY642072	I		ND
P24	−	II	AY642046	AY642058	II	AY644406	II
PV37	+	I		AY642068	I	AY644414	I
PV58	+	I		AY642073	I		ND
PV66	−	Atypical	AY642041	AY642062	I	AY644410	I
PV93	−	Atypical	AY642043	AY642063	I	AY644411	I
P135	+	I		AY642074	I		ND
P136	+	I		AY642075	I		ND
Skin incision							
JMK9	+	Atypical		AY642095	I	AY644421	I
MMG9	+	Atypical		AY642094	I	AY644420	I
TON9	+	Atypical		AY642096	I	AY644422	I
WMK9	+	Atypical		AY642093	I	AY644419	I
RM9	+	Atypical	AY642050	AY642070	I	AY644416	I
Dental[b]							
W513	+	I		AY642079	I		ND
W633	+	I		AY642080	I		ND
W891	+	I		AY642081	I		ND
W1034	+	I		AY642076	I		ND
W1392	+	Atypical	AY642051	AY642059	I	AY644407	I
W1973	+	I		AY642077	I		ND
W1998	+	Atypical		AY642078	I	AYY644417	I
Prosthesis[c]							
AT1[d]	+	I	AY642048	AY642082	I		ND
ATB1	−	II		AY642087	II		ND
CK17	+	Atypical		AY642092	I	AY644418	I
DW1[d]	−	II		AY642083	II		ND
ED1	−	II		AY642069	II	AY644415	II
ED2[d]	−	II	AY642052	AY642084	II		ND
ET1	−	II		AY642085	II		ND
JP1B	−	II	AY642053	AY642065	II	AY644413	II
JR2	+	I		AY642086	I		ND
KC1	−	II		AY642066	II		ND
L1958	+	I	AY642042	AY642056	I	AY644404	I
LED2	+	Atypical	AY642049	AY642060	I	AY644408	I
RB1B	−	II		AY642067	II		ND
RM1	+	I		AY642097	I	AY644423	I
RM4	−	II	AY642047	AY642057	II	AY644405	II
SG2	−	II	AY642045	AY642064	II	AY644412	II
SR2	−	II		AY642088	II		ND
TFJ2	−	II		AY642089	II		ND
WD1	−	II	AY642054	AY642090	II		ND
WD2	−	II		AY642091	II		ND

[a] Some isolates gave atypical labeling with MAbs QUBPa1 and QUBPa2.
[b] Isolates recovered from patients with periodontitis, pericoronitis, and endodontic infections.
[c] Isolates recovered directly from prosthetic hips or associated bone and tissue samples.
[d] Isolates used for the generation of MAbs QUBPa1 and QUBPa2.
[e] ND, not determined.

also characteristic of the *P. acnes* type I or II reference strain (NCTC 737 or NCTC 10390). All isolates conclusively identified as type II by use of our MAbs did not ferment sorbitol.

Nucleotide sequence analysis. All nucleotide sequencing was carried out on pooled PCR products to eliminate potential inaccuracies that can arise in the sequencing of individually cloned PCR products. A total of 15 *P. acnes* isolates, assigned to type I or type II on the basis of both MAb labeling and fermentation profiles, were selected for systematic analysis of 16S rRNA (Table 1). By using the universal bacterial 16S rRNA-based primers UFPL and URPL, a 1,484-bp product was amplified from all strains. Nucleotide sequence analysis with UFPL and URPL, as well as internal 16S rRNA-based primers, produced a complete sequence. Sequence analysis of

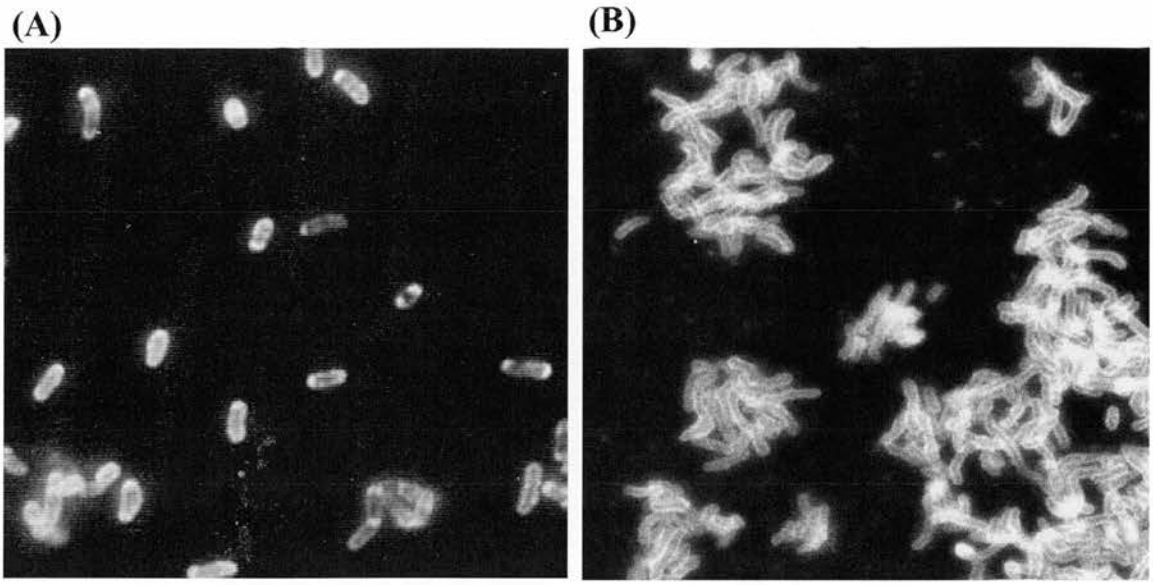

FIG. 1. Micrographs of *P. acnes* immunolabeled with a mouse MAb and an FITC-conjugated anti-mouse antibody. Bacteria were counterstained with Evans blue and viewed with a combined red/green filter (×100 objective). (A) Reference strain NCTC 737 (type I) reacted with MAb QUBPa1; (B) reference strain NCTC 10390 (type II) reacted with MAb QUBPa2.

the 16S rRNA gene from *P. acnes* NCTC 737 was carried out as an internal control. The resulting sequence was identical to the previously published 16S rRNA gene for this strain (GenBank accession no. AB042288). Multiple sequence alignments of the 1,484-bp 16S rRNA genes from *P. acnes* types I and II ($n = 15$) revealed >99.5% sequence identity. Only one type-specific polymorphism, at position 827 (numbering corresponds to GenBank accession no. AB042288), was observed. This corresponded to the nucleotide T in type I strains and C in type II strains.

Phylogenetic analyses of *P. acnes* types I and II based on the *recA* gene and a putative hemolysin gene (*tly*), both of which we identified from the draft genome sequence of NCTC 737 (property of Corixa Corporation, Seattle, Wash.), were also carried out. By use of PAR-1 and PAR-2, 1,201-bp products were amplified from the *recA* genes of NCTC 737 and NCTC 10390 as representatives of the two *P. acnes* types. Sequence analysis of *P. acnes* NCTC 737 served as an internal control. The resulting *recA* sequence for our NCTC 737 internal-control strain was identical to the NCTC 737 genome sequence for *recA* (GenBank accession no. AY642055). Alignment of the 1,047-bp *recA* genes from NCTC 737 and NCTC 10390 revealed 10 type-specific differences (99% sequence identity). To facilitate phylogenetic analysis, we sequenced the *recA* gene from a further 41 *P. acnes* strains isolated from different clinical sources and selected to represent types I and II (Table 1).

By use of PAT-1 and PAT-2, 909-bp products were amplified from the putative hemolysin genes of NCTC 737 and NCTC 10390. The resulting *tly* sequence for our NCTC 737 internal-control strain was identical to the NCTC 737 genome sequence for *tly* (GenBank accession no. AY527219). Alignment of the 777-bp *tly* genes from NCTC 737 and NCTC 10390 revealed 18

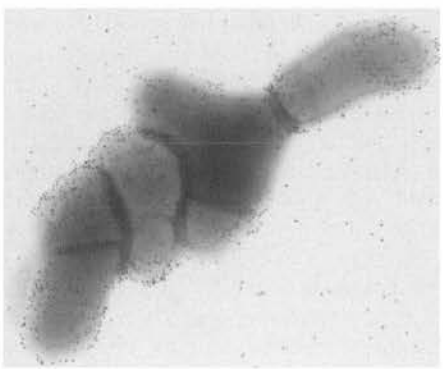

FIG. 2. Electron micrograph of *P. acnes* NCTC 737 (type I), immunolabeled with MAb QUBPa1 and an anti-mouse IgG conjugated with 15-nm-diameter gold particles, and negatively stained with ammonium molybdate. Note labeling at the septa and apices of cells.

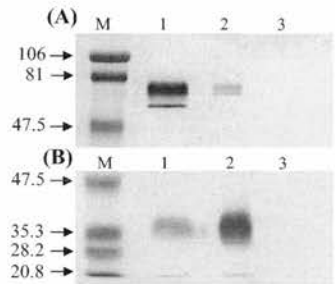

FIG. 3. Immunoblots of *P. acnes* with MAbs QUBPa1 and QUBPa2. (A) Labeling with QUBPa1. Lanes: M, molecular weight markers (in thousands); 1, whole-cell sonicate preparation of NCTC 737; 2, purified cell wall sonicate preparation of NCTC 737; 3, whole-cell sonicate preparation of NCTC 10390. (B) Labeling with QUBPa2. Lanes: M, molecular weight markers (in thousands); 1, whole-cell sonicate preparation of NCTC 10390; 2, purified cell wall sonicate preparation of NCTC 10390; 3, whole-cell sonicate preparation of NCTC 737.

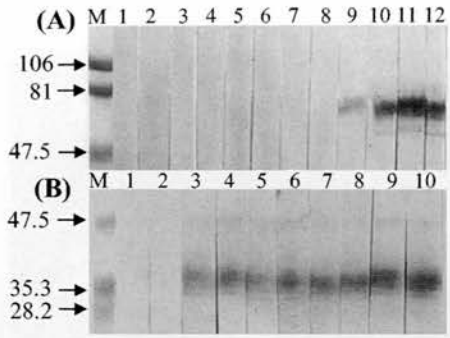

FIG. 4. Effects of proteinase K and sodium-*meta*-periodate treatments on *P. acnes* reactivities with MAbs QUBPa1 and QUBPa2. (A) QUBPa1 reactivity with proteinase K-treated whole-cell sonicate preparations. Lane M, molecular weight markers (in thousands). Lanes 1 to 12 contain the following concentrations (in milligrams per milliliter) of proteinase K: 10^{-1} (lane 1), 5×10^{-2} (lane 2), 2.5×10^{-2} (lane 3), 1.25×10^{-2} (lane 4), 6.25×10^{-3} (lane 5), 3.12×10^{-3} (lane 6), 1.56×10^{-3} (lane 7), 7.81×10^{-4} (lane 8), 3.90×10^{-4} (lane 9), 1.95×10^{-4} (lane 10), 9.76×10^{-5} (lane 11), and 4.88×10^{-5} (lane 12). (B) QUBPa2 reactivity with sodium-*meta*-periodate-treated whole-cell sonicate preparations. Lane M, molecular weight markers (in thousands). Lanes 1 to 10 contain the following molar concentrations of sodium-*meta*-periodate: 10^{-1} (lane 1), 5×10^{-2} (lane 2), 2.5×10^{-2} (lane 3), 1.25×10^{-2} (lane 4), 6.25×10^{-3} (lane 5), 3.12×10^{-3} (lane 6), 1.56×10^{-3} (lane 7), 7.81×10^{-4} (lane 8), 3.90×10^{-4} (lane 9), and 1.95×10^{-4} (lane 10).

type-specific differences (98% sequence identity). For phylogenetic analysis, we sequenced the *tly* gene from a further 19 *P. acnes* strains isolated from different sources and selected to represent types I and II (Table 1).

Phylogenetic analysis of *recA* and *tly*. Phylogenetic trees of *P. acnes* based on *recA* and *tly* gene sequences were constructed. The *recA* and *tly* gene sequences from *Helicobacter pylori* (GenBank accession no. U13756 and AE000615) and *Streptococcus agalactiae* (GenBank accession no. AF326345 and NC_004116) were used as outgroups for our phylogenetic analysis, which was performed using the maximum-parsimony and neighbor-joining methods. After 100 bootstrapping replications, the *recA* and *tly* consensus trees derived by using the two methods gave the same topology, with type I and II strains forming distinct cluster groups. Only the consensus trees obtained by using the neighbor-joining method are shown (Fig. 5). Both the *recA* and *tly* phylogenies of *P. acnes* were highly distinct from unrelated species selected as outgroups for the trees (bootstrap values, 100%). Phylogenetic trees of *recA* and *tly* based on protein translation of each nucleotide sequence revealed the same clustering of types I and II as distinct phylogenetic groups, even though the protein sequences were less discriminatory than the nucleotide sequences (data not shown). Phylogenetic analysis of the *recA* and *tly* sequences from all 11 isolates with atypical MAb labeling revealed the organisms to belong to type I. On both trees, all nine atypical type I isolates that were weakly positive with QUBPa2 clustered separately from other type I strains.

TABLE 2. Reactivities of *P. acnes* isolates with MAbs QUBPa1 and QUBPa2

Source	No. of isolates with the following reactivity (% sorbitol fermenters):			Total
	QUBPa1	QUBPa2	Atypical	
Prosthetic hips				
Implant	19 (79)	20 (0)	1 (100)	40
Bone[a]	5 (80)	1 (0)	0	6
Tissue[a]	29 (83)	8 (0)	1 (100)	38
Skin				
Acne	19 (100)	1 (0)	2 (0)	22
Incision	9 (100)	4 (0)	5 (100)	18
Dental	6 (100)	0	2 (100)	8
Total	87 (88.5)	34 (0)	11 (82)	132

[a] Bone and tissue associated with failed prosthetic hip implant.

DISCUSSION

Using IFM, we identified two MAbs, designated QUBPa1 and QUBPa2, which were specific for cell surface epitopes of the reference strains NCTC 737 and NCTC 10390, respectively. The MAbs were highly specific and did not react with bacteria from a range of related and unrelated genera. Due to their specificity, these MAbs may prove especially valuable for direct IFM-based detection of *P. acnes* types I and II from clinical specimens, such as large aggregates of *P. acnes* biofilm dislodged by ultrasound from infected prosthetic hip implants (33).

Immunogold labeling and IFM analysis revealed that QUBPa1 was specific for an antigen that accumulates at the septa and apices of type I cells. Immunoblotting experiments with whole-cell preparations of NCTC 737 revealed labeling of two bands at 61 and 68 kDa. With a purified cell wall preparation, the 61-kDa component was absent or reduced in quantity, findings that may be related to dissociation from the cell envelope. Considering that reactivity with QUBPa1 was abolished after treatment of the antigen with proteinase K, but not after sodium-*meta*-periodate treatment, it appears that the bands detected were proteinaceous. Although MAbs have specificity for a single epitope, it is not uncommon for multiple bands to be labeled on Western blots (9). Multiple bands may result from molecules that share the same epitope or from an antigen that undergoes posttranslational modification or proteolytic cleavage. For example, we have identified another cell surface protein in *P. acnes* that has a labile N-terminal region and produces components of different molecular masses after SDS-PAGE and immunoblotting with MAbs (unpublished data). MAb QUBPa2 labeled the cell surfaces of type II strains. Immunoblotting revealed a diffuse band(s) between 30 and 40 kDa and a single band at 21 kDa. The immunoreactivity of these bands was abolished after treatment with sodium-*meta*-periodate but not proteinase K, providing evidence that these antigens share a carbohydrate or glycolipid-containing epitope. The diffuse or smeared band(s) detected at 30 to 40 kDa is, therefore, likely to represent a polymeric antigen with a varying number of saccharide units. Previous immunodiffusion experiments with polyclonal antisera against *P. acnes* have also concluded that cell wall polysaccharides of the organism can act as antigenic determinants (3, 4). A key distinction between *P. acnes* types I and II is the absence of galactose in

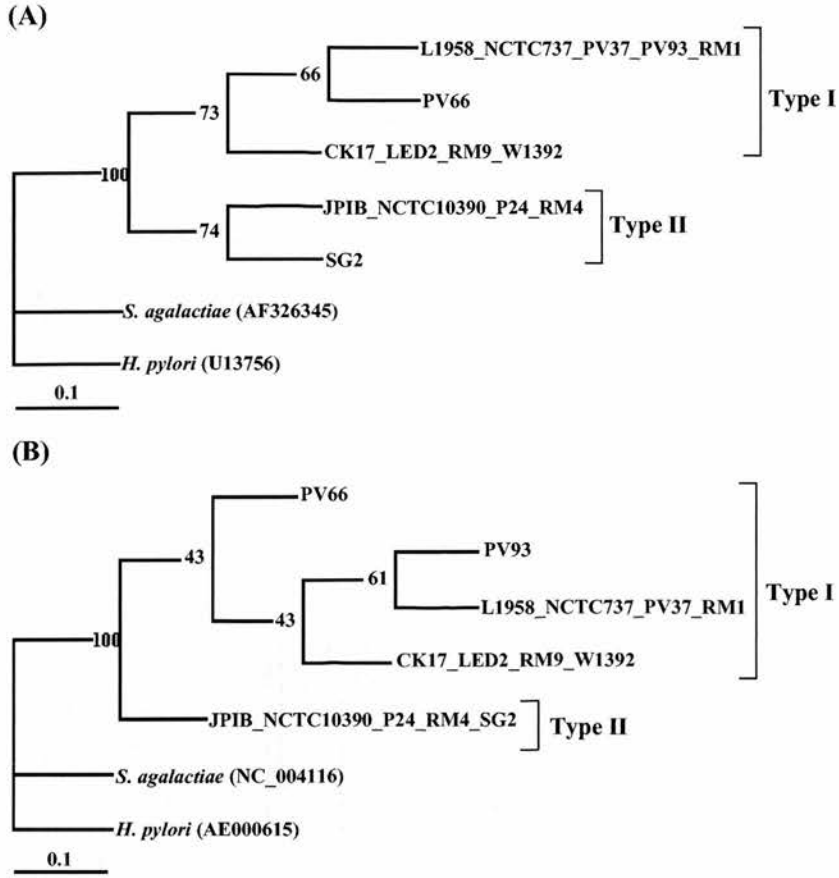

FIG. 5. Phylogenetic trees of *P. acnes* based on the complete *recA* (A) and *tly* (B) gene sequences. Multiple sequence alignments were performed on these genes and the published sequences for *H. pylori* (U13756 and AE000615) and *Streptococcus agalactiae* (AF326345 and NC_004116). The resulting phylogenetic trees were rooted with the *H. pylori* sequences. Bootstrapping resampling statistics were applied to the trees (100 data sets), and bootstrap values are shown at each node of the tree. The type status for the different strains analyzed is given on the right. Phylogenetic analysis was performed on a selection of isolates chosen to represent different nucleotide sequences.

the cell envelopes of type II strains (14), as well as differences in lipoglycan composition (40). Whether these features relate to the epitope recognized by QUBPa2 still remains to be determined. Currently, work to purify and further characterize the type I and II antigens is ongoing in our laboratory.

Using our MAbs, we conducted a preliminary epidemiological study of *P. acnes* isolates for type status (Table 2). Previously, by adopting strict anaerobic protocols and including a mild ultrasonication step to remove adherent bacteria growing as a biofilm, we isolated *P. acnes* as frequently as *Staphylococcus* spp. from implants and associated tissue of patients undergoing revision hip surgery (33, 34). In this study, analysis of *P. acnes* isolates recovered directly from prosthetic hip implants revealed approximately equal numbers of type I and II strains, while isolates from associated bone and tissue samples were predominantly of type I. Isolates recovered from patients with acne and dental infections, and skin isolates from surgical incision sites, were also found to be predominantly type I. Further studies with a larger group of isolates from similar patients will be valuable for confirming this distribution of types. Because skin and oral isolates appear to belong predominantly to type I, yet a significant number of type II isolates were recovered from prosthetic hip implants, it will be important to identify the exact source of these type II organisms. Analysis of *P. acnes* isolates from other sites may help address this issue and provide a better understanding of infection routes for prosthetic infections. Also, a more extensive study of type I and II distribution among oral isolates may prove particularly valuable, because dental manipulations and dentogingival infections, such as dental caries and periodontal disease, have been implicated in prosthetic joint infection through hematogenous spread (19, 30). *P. acnes* in particular has been reported to constitute up to 9.0% of the dental microbiota, and studies to recover obligate anaerobes from carious dentin indicated that approximately 20% of all isolates were *P. acnes* (1, 12).

Investigation of the phylogenetic relationship between *P. acnes* types I and II revealed only one type-specific base difference in the 16S rRNA gene sequence, highlighting the close relationship between the two groups. Although sequence analysis of the 16S rRNA gene is widely recognized as a powerful method for investigating the phylogenetic relationship between bacteria, it may not be the most appropriate technique for distinguishing between related members of a genus or species (36). In contrast, nonribosomal housekeeping genes (e.g., *recA*, ATP-synthase, and GroEL) have frequently been found to give

better insights into the phylogenetic relationship that exists between closely related organisms (15, 22, 37). In particular, analysis of the *recA* gene has been valuable for the separation of closely related species, and bacterial classifications based on *recA* have proved to be consistent with those obtained by using rRNA genes (6). As a consequence, we investigated the relationship between *P. acnes* types I and II based on *recA*. Furthermore, we also examined the phylogenies of both types by analyzing a putative *P. acnes* hemolysin gene, known as *tly*, which we also identified from the NCTC 737 genome sequence.

Sequence analysis of the *recA* and *tly* genes from a selection of *P. acnes* strains revealed a significantly greater number of conserved type-specific base differences than sequence analysis of the 16S rRNA locus. Phylogenetic analysis of both the *recA* and *tly* sequences revealed that types I and II represent phylogenetically distinct groups, further demonstrating the benefits of protein-encoding DNA for systematic analysis. The agreement of the *tly* phylogenetic tree with that of *recA* also demonstrated the value of taxon-specific genes in phylogenetic investigations. Such genes are under selective pressures different from those for universal housekeeping genes and therefore may be less well conserved, giving rise to better phylogenetic resolution of closely related organisms. Recently, analysis of 46 *P. acnes* isolates by random amplification of polymorphic DNA (RAPD) revealed two distinct genomic profiles (28). Although the type status of these various isolates was not determined, RAPD profiles from the type I and II reference strains, NCTC 737 and NCTC 10390, respectively, did match the two RAPD lineages of these *P. acnes* isolates, providing further evidence for the distinct genomic natures of types I and II. Studies of *Burkholderia cenocepacia* (genomovar III), which causes infections among patients with cystic fibrosis, has also revealed the presence of two phylogenetically distinct *recA* lineages (known as III-A and III-B) (24). Epidemiological investigations have shown that strains of these two lineages not only differ in their distribution among infected patients in different geographical localities (21, 29) but also display differences in the presence of specific virulence factors (24). Two further phylogenetically distinct *recA* lineages (III-C and III-D) of *B. cenocepacia* have also been described recently (35). Whether the different *recA* lineages of *P. acnes* differ significantly with respect to virulence awaits further research.

Although the majority of *P. acnes* isolates (92%) could be conclusively identified as type I or II based on MAb immunoreactivity, which was confirmed by DNA sequencing of a representative selection of strains, we did identify nine atypical strains that fermented sorbitol yet did not react with QUBPa1 but showed weak reactions with QUBPa2. In addition, we also detected two isolates that reacted with both MAbs, although labeling was strong with one MAb and poor with the other. *recA* and *tly* sequencing of these 11 atypical *P. acnes* isolates identified them as type I. In the *recA* and *tly* phylogenetic trees, all nine atypical type I isolates that were weakly positive with QUBPa2 clustered on a separate branch from other type I strains. These organisms may belong to a further phylogenetic subdivision within type I. It will be necessary, however, to perform molecular typing experiments, such as pulsed-field gel electrophoresis, to confirm that these isolates do indeed represent different strains and not a single atypical strain or clone isolated from different patients. We have also found that these atypical type I isolates express high levels of a surface and secreted antigen that is normally present in large quantities only among type II organisms (unpublished data), thus further demonstrating their distinct nature. The atypical reactions of PV66 and PV93 with QUBPa1 and QUBPa2 suggest that their exact relationship to other type I strains may warrant further investigation. Indeed, although earlier studies indicated that *P. acnes* types I and II had similar GC contents and DNA homologies (14), a more detailed polyphasic taxonomic study of these various *P. acnes* groups may now be appropriate in the light of our phylogenetic data.

The identification of type-specific nucleotide differences between types I and II has also revealed that DNA sequencing can be used as an accurate method for the identification of *P. acnes* types. Currently, we are investigating more practical DNA-based methods, such as restriction fragment polymorphism analysis and/or the use of type-specific primers, which could be used as a complement to DNA sequencing and MAb labeling.

In conclusion, phylogenetic analysis has revealed that *P. acnes* types I and II represent distinct lineages. Therefore, the well-described antigenic and biochemical differences between types I and II reflect deeper differences in their phylogeny. Improvements in the identification of *P. acnes* types I and II, and a better understanding of the organism's phylogeny, will facilitate the study of *P. acnes* epidemiology and pathogenesis.

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Extensive DNA inversions in the *B. fragilis* genome control variable gene expression.
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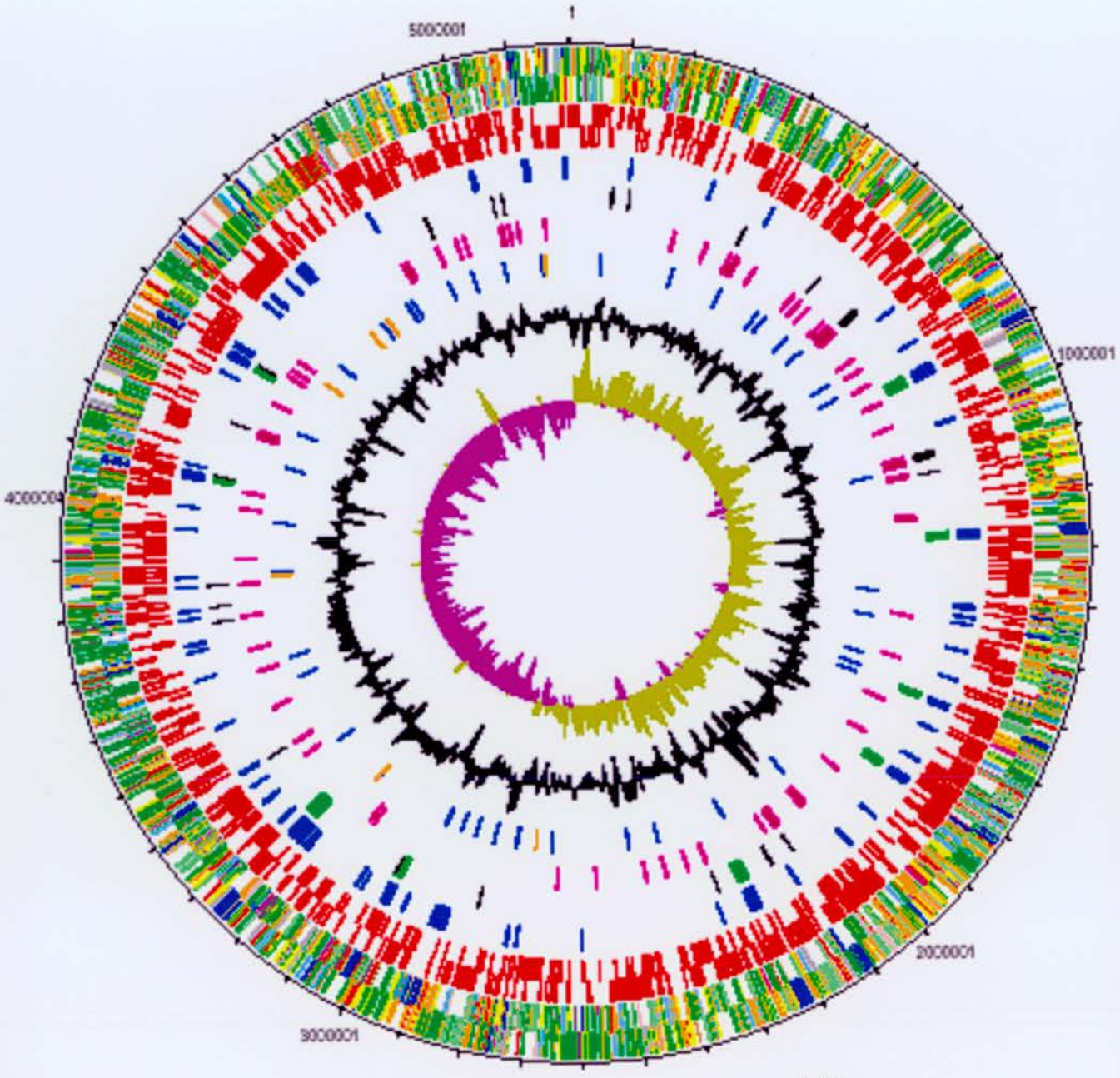
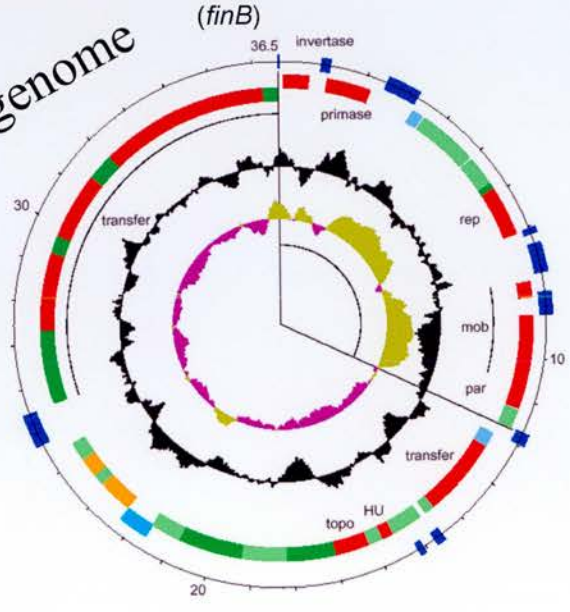


Bacteroides fragilis NCTC9343 genome



Spiroplasma was fully operational. First, crosses involving heterozygous mothers, where male-killing was complete, were conducted concurrently with those using homozygous mothers, and the females in these crosses were siblings from the same vials. Second, in each cross and vial where homozygous males survived, heterozygous males (with wild-type function) still died. Finally, F_1 females derived from these crosses, when mated to wild-type males, produced a full, female-biased sex ratio.

In the case of *msl*-2, where there is no maternal supply of MSL-2, survival of homozygous sons was observed for both homozygous and heterozygous mothers for the case of the mutation *msl-2*^{g134} (Fig. 1). For the case of *msl-2* ^{g227}, no male progeny were observed from infected females (table S5). This mutation does not rescue males in our assay, probably because the two mutations have different effects on *msl-2* expression. The *msl-2*^{g134} allele prevents formation of MSL-2 protein, whereas *msl-2* ^{g227} potentially encodes the RING-finger element of a truncated MSL-2 protein (*14*).

Thus, absence or reduced function of any of the proteins of the DCC can reduce the efficiency of male killing, and a functional DCC is required for male killing by *S. poulsonii*. The fact that the genes mediating this process in *Drosophila* have been well studied can be exploited to yield further insights into the mechanism of male killing.

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Supporting Online Material

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References and Notes

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Extensive DNA Inversions in the *B. fragilis* Genome Control Variable Gene Expression

Ana M. Cerdeño-Tárraga,[1] Sheila Patrick,[2]* Lisa C. Crossman,[1] Garry Blakely,[3] Val Abratt,[4] Nicola Lennard,[1] Ian Poxton,[5] Brian Duerden,[6] Barbara Harris,[1] Mike A. Quail,[1] Andrew Barron,[1] Louise Clark,[1] Craig Corton,[1] Jonathan Doggett,[1] Matthew T. G. Holden,[1] Natasha Larke,[1] Alexandra Line,[1] Angela Lord,[1] Halina Norbertczak,[1] Doug Ormond,[1] Claire Price,[1] Ester Rabbinowitsch,[1] John Woodward,[1] Bart Barrell,[1] Julian Parkhill[1]*

The obligately anaerobic bacterium *Bacteroides fragilis*, an opportunistic pathogen and inhabitant of the normal human colonic microbiota, exhibits considerable within-strain phase and antigenic variation of surface components. The complete genome sequence has revealed an unusual breadth (in number and in effect) of DNA inversion events that potentially control expression of many different components, including surface and secreted components, regulatory molecules, and restriction-modification proteins. Invertible promoters of two different types (12 group 1 and 11 group 2) were identified. One group has inversion crossover (*fix*) sites similar to the *hix* sites of *Salmonella typhimurium*. There are also four independent intergenic shufflons that potentially alter the expression and function of varied genes. The composition of the 10 different polysaccharide biosynthesis gene clusters identified (7 with associated invertible promoters) suggests a mechanism of synthesis similar to the O-antigen capsules of *Escherichia coli*.

Bacteroides fragilis is the major obligately anaerobic Gram negative bacterium isolated from abscesses, soft tissue infections, and bacteraemias that arise from contamination of normally uncolonized body sites by bacteria from the resident gastrointestinal (GI) microbiota. Putative virulence attributes of *B. fragilis* include attachment mechanisms, aerotolerance, extracellular enzyme production, and resistance to complement-mediated killing and phagocytosis [reviewed in (*1*)]. The lipopolysaccharide of *B. fragilis* triggers inflammatory events via the Toll-like receptor 2 (TLR2) and is likely to be involved in systemic inflammatory response syndrome caused by GI tract bacteria (*2*). *B. fragilis* itself only accounts for between 4 and 13% of the normal human fecal microbiota but is present in 63 to 80% of *Bacteroides* infections. In contrast, the related *B. thetaiotaomicron* accounts for between 15 and 29% of the fecal microbiota but is associated with only 13 to 17% of infection cases. *B. fragilis* is capable of a high amount of within-strain phase and antigenic variation of surface components. A single strain of *B. fragilis* may reversibly produce three different encapsulating surface structures: the large capsule and the small capsule, both visible by light microscopy, and an electron-dense layer (EDL) visible by electron microscopy (*3*). In addition, reversible within-strain antigenic variation of multiple antigenically distinct high molecular mass polysaccharides and other components is evident (*4*). Before the advent of the genome sequencing program, the potential mechanisms generating this variation were unknown. We determined the complete genome sequence of the nonenterotoxin-producing DNA homology group I *B. fragilis*, strain NCTC 9343.

The genome of *B. fragilis* NCTC 9343 contains a single circular chromosome of 5,205,140 base pairs (bp) predicted to encode

[1]Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, UK. [2]Department of Microbiology and Immunobiology, School of Medicine, Queen's University of Belfast, Grosvenor Road, Belfast, BT12 6BN, UK. [3]Institute of Cell and Molecular Biology, University of Edinburgh, Darwin Building, Kings Buildings, Edinburgh EH9 3JR, UK. [4]Department of Molecular and Cell Biology, University of Cape Town, Private Bag Rondebosch 7701, South Africa. [5]Division of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, UK. [6]Department of Medical Microbiology, University of Wales College of Medicine, Heath Park, Cardiff, CF14 4XN, UK.

*To whom correspondence should be addressed. E-mail: parkhill@sanger.ac.uk (J.P.); s.patrick@qub.ac.uk (S.P.)

4274 genes and a plasmid, pBF9343 (fig. S1 and table S1). During the assembly of shotgun data, particular regions could not be resolved because certain segments of the sequence were present in two alternative orientations. This indicated that specific inversions of these sequences occurred at a high frequency within the clonal growth of bacteria used for DNA isolation. These fragilis invertible (*fin*) regions can be grouped on the basis of the inverted repeat sequences that flank them. Twelve regions (table S2A, group 1) shown to be invertible or with sequence similarity are flanked by inverted repeats, designated fragilis inversion crossover (*fix*) sites, similar to those acted on by the *Salmonella* Hin DNA invertase (*5*). All of these invertible regions contain a consensus promoter, suggesting that they control the expression of downstream genes. Seven *fin* regions (average length of 226 bp) were found upstream of 7 of the 10 polysaccharide biosynthesis gene clusters (table S3), immediately suggesting a mechanism for the observed antigenic variation. The orientation of specific promoters can be correlated experimentally with expression of specific polysaccharides (*6*), an observation confirmed in independent experiments (*5*). The remaining five related *fin* regions in group 1 are 161 bp in length and are associated with a variety of other putative proteins (table S2A). We identified two serine site-specific DNA invertases similar to Hin in the genome, FinA (BF2779), chromosomally located but not near an invertible region, and FinB (pBF9343.01), on the plasmid. The role of FinA (Mpi) in the inversion of these segments has been demonstrated (*7*), and the plasmid-encoded FinB binds to *fix* sites (*5*). In total, the genome encodes 30 enzymes potentially capable of site-specific DNA inversion: 26 tyrosine recombinases (integrase family), 3 serine recombinases (resolvase-invertase family), and 1 Piv-like transposase-invertase (IS110 family).

A further 11 *fin* promoter regions (average length of 370 bp) are different from the *hin*-like group 1 regions and more heterogeneous in nature (table S2A, group 2). The inverted repeats that flank these regions and contain the sites of strand exchange (group 2 *fix* sites) are different from the *hix*-like regions, indicating that they are acted on by a different recombinase. These predominantly control the expression of a family of outer membrane proteins, and some might also drive the expression of divergent genes with diverse functions [Supporting Online Material (SOM) Text]. The use of DNA inversion by *B. fragilis* goes beyond the control of promoter sequences. Several more complex inversion events, or intergenic shufflons, that involve the inversion of complete and partial coding sequences were observed in the shotgun sequence. One example, whereby DNA inversion brings silent gene segments into an expression site, is the two-domain specificity protein of a type-I restriction-modification system (BF1839) (table S2B, IR-BB). Each domain in such proteins is responsible for recognizing half of the two-part DNA binding site. Just after the start codon and between the two domains of BF1839 are independent inverted repeats that are unrelated to group 1 or 2 *fix* sites, both of which are present in similar positions in the downstream convergent gene BF1842, which does not have an appropriate start codon (Fig. 1A). Independent recombination events between these inverted repeats would produce four different specificity proteins recognizing four different DNA sequences. Between these genes are two further gene cassettes, each of which encodes one C-terminal recognition domain. At the 5′ end of these cassettes are two further, different inverted repeats that allow either of the cassettes to be exchanged with the C-terminal domain of the adjacent gene (BF1838 or BF1842), increasing the number of potential recognition specificities to eight. Three potential recombinases encoded nearby (BF1833, 1843, and 1845) may be involved in this system. A similar, although less complex, variable restriction-modification system has been described in *Mycoplasma pulmonis* (*8*). Three further independent intergenic shufflons, acting on outer membrane proteins and a signal transduction system, were observed in the shotgun sequence (Fig. 1, table S2B, and SOM Text). Other intergenomic inversions (IR-Q, IR-R, and IR-S) (table S3) involve the inversion of complete coding sequences, often reorientating them with or against the apparent direction of transcription of surrounding genes. These may also affect the transcription levels of the genes within these regions.

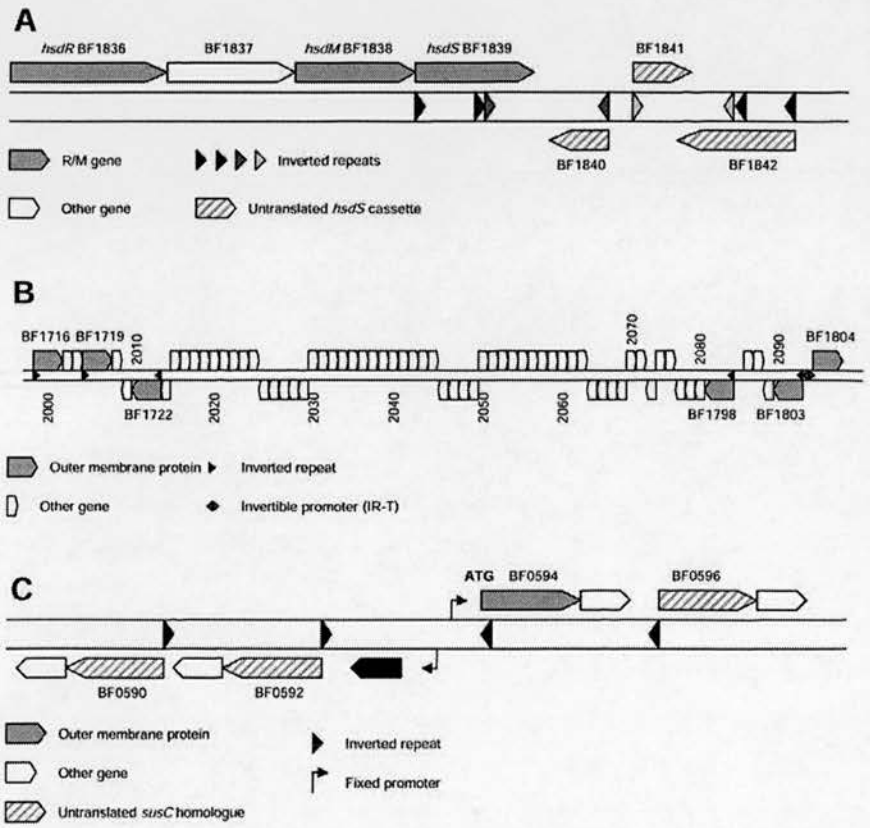

Fig. 1. Examples of invertible regions in the *B. fragilis* genome (*18*). (**A**) Restriction modification intergenic shufflon: restriction-modification (R/M) complex genes, gray boxes; other genes, open boxes; potential *hsdS* DNA binding modules, hatched boxes; different inverted repeats at the inversion ends, light gray triangles. (**B**) Inversion of large segments of DNA through large inverted repeats (black triangles) brings alternative outer membrane protein genes (gray boxes) downstream of an invertible promoter (gray diamond). (**C**) Local inversion through inverted repeats (black triangles) fuses silent alternative outer membrane protein gene cassettes (hatched boxes) to a fixed promoter and translation start.

Comparison of the *B. fragilis* genome with the recently sequenced *B. thetaiotaomicron* strain VPI 5482 (ATCC 29148) (*9*) reveals that there are no orthologous variable promoters or indeed operons driven by them. *B. thetaiotaomicron* does encode some variable systems (*9*), but they are unique to that

organism and considerably less numerous than those in *B. fragilis*. This enhanced potential for variation and other genomic differences (SOM Text) may explain in part why *B. fragilis* is isolated more frequently from infection than *B. thetaiotaomicron*.

Surface polysaccharides are involved in establishing abscess formation (*10*). Ten separate gene clusters potentially involved in polysaccharide synthesis are evident in the genome sequence (table S3). The polysaccharide gene clusters A to H have genes similar to *wzx* and *wzy* that are involved in transfer of linked sugar repeats across the cytoplasmic membrane and repeat unit polymerization, respectively, but are lacking in genes associated with the export of polymer across the outer membrane. This suggests that these gene clusters are similar to the *Escherichia coli* group 4 O-antigen capsules (*11*) and is in keeping with the characteristic heterogeneity of the polysaccharide chain length after SDS–polyacrylamide gel electrophoresis (PAGE) and the EDL phenotype (*12*). A gene with some similarity to *E. coli wzz* (BF1708) that determines chain length is located within polysaccharide gene cluster J. Variation in the expression of BF1708 may explain the varying reports of presence (*13*) or absence (*14*) of repeating O-antigen units after PAGE.

Phase variation controlled by DNA inversion events has been reported in several other bacteria. For example, *Salmonella typhimurium* regulates the expression of a flagellar protein by using a single invertible promoter (*15*), and *E. coli* plasmids use shufflons to express one of several variant pilus proteins (*16*). Different species of *Mycoplasma* have been shown to regulate the expression of a number of surface proteins by using invertible promoters (*17*) or to use a shufflon system to express variable surface proteins (*8*). However, in each of these cases, the use of these mechanisms is restricted to a single system or class of surface molecules. As described here, *B. fragilis* uses DNA inversion to control a larger number or greater breadth of systems than in any other organism described to date, including surface proteins, polysaccharides, and regulatory systems. This may be related to its niche as a commensal and opportunistic pathogen, because the resulting diversity in surface structures could increase both immune invasion and the ability to colonize novel sites.

Note added in proof: The genome sequence of another strain of *B. fragilis* has recently been published (*19*), and the analysis is in general agreement with that presented here.

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Supporting Online Material

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Requirement for Caspase-8 in NF-κB Activation by Antigen Receptor

Helen Su,[1]* Nicolas Bidère,[1]* Lixin Zheng,[1] Alan Cubre,[1] Keiko Sakai,[1] Janet Dale,[2] Leonardo Salmena,[3] Razqallah Hakem,[3] Stephen Straus,[2] Michael Lenardo[1]†

Caspase-8, a proapoptotic protease, has an essential role in lymphocyte activation and protective immunity. We show that caspase-8 deficiency (CED) in humans and mice specifically abolishes activation of the transcription factor nuclear factor κB (NF-κB) after stimulation through antigen receptors, Fc receptors, or Toll-like receptor 4 in T, B, and natural killer cells. Caspase-8 also causes the αβ complex of the inhibitor of NF-κB kinase (IKK) to associate with the upstream Bcl10-MALT1 (mucosa-associated lymphatic tissue) adapter complex. Recruitment of the IKKα,β complex, its activation, and the nuclear translocation of NF-κB require enzyme activity of full-length caspase-8. These findings thus explain the paradoxical association of defective apoptosis and combined immunodeficiency in human CED.

The intracellular aspartate-specific cysteine protease caspase-8 initiates death receptor signaling for apoptosis (*1*). Recruitment into the death-signaling complex induces procaspase-8 oligomerization, followed by full processing into a highly active soluble tetramer (*2*, *3*). However, caspase-8 is also essential for lymphocyte activation and protective immunity in mice and humans (*4*, *5*). Patients with caspase-8 deficiency (CED) have defective apoptosis and immunodeficiency due to impaired activation of T, B, and natural killer (NK) lymphocytes (*4*).

Human peripheral blood leukocytes (PBLs) or mouse T cells treated with the pan-caspase inhibitor benzyloxycarbonylvalyl-alanyl–aspartic acid (*O*-methyl)–fluoro-methylketone (zVAD) have reduced antigen receptor–induced expression of interleukin-2 (IL-2) and its receptor subunit CD25 (*4*, *6*, *7*). Because nuclear factor κB (NF-κB) is required for IL-2 and CD25 gene transcription as well as lymphocyte activation (*8*, *9*), we examined this transcription factor in cells exposed to caspase inhibitor. NF-κB family members—Rel (c-*rel*), RelA (p65), RelB, NF-κB1 (p105/50), and NF-κB2 (p100/52)—regulate gene transcription as dimers (*8*, *9*).

[1]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA. [2]Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA. [3]Ontario Cancer Institute, University of Toronto, Toronto, Ontario M5G 2C1, Canada.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: lenardo@nih.gov

Supporting Online Material

Materials and Methods

This sequence strain NCTC 9343 was originally isolated from an abdominal infection at St Bartholomew's Hospital, London in 1955. The culture from which DNA was produced for the genomic sequencing was enriched for the EDL phase, with minimum subculture from an early freeze dried stock culture, but remained antigenically mixed. Bacteria were grown in defined medium broth (*1*) in an anaerobic cabinet (MACS Anaerobic Workstation Don Whitley Scientific, Shipley, UK; 80% N2, 10% CO2 and 10% H2). Percoll density gradient enrichment was used to obtain populations that were non-capsulate by light microscopy as described previously (*2*).

DNA was isolated using a modification of the basic protocol for preparation of genomic DNA from bacteria detailed in Ausubel *et al.* (*3*). In brief, bacterial cells were lysed in 10 mM TrisHCl 1mM EDTA buffer (pH 8.0) containing SDS (0.5%), lysozyme (4mg/ml) and proteinase K (0.1mg/ml). Polysaccharide was precipitated using cetyltrimethylammonium bromide (CTAB) and DNA extracted with chloroform: isoamyl alcohol and phenol:choloroform:isoamyl alcohol mixtures. DNA was precipitated using isopropanol, spooled out with a glass rod and washed in ethanol. The initial genome assembly was obtained from 94,563 paired end sequences (giving 10-fold coverage) derived from four pUC18 genomic shotgun libraries (with insert sizes ranging from 2.0 to 4.0 kb) using big-dye terminator chemistry on ABI3700 automated sequencers. This was supplemented with 4,991 end-reads from an m13mp18 library with an insert size of 2.0-4.0 kb; 3,388 paired-end sequences

from a pBACe3.6 library with insert sizes of 10-18 kb (a clone coverage of 4.56-fold) were used as a scaffold. All identified repeats were bridged by read-pairs or end-sequenced PCR products. A further 4,594 sequencing reads were generated during finishing. The sequences were assembled, finished and annotated as described previously (*4*), using Artemis (*5*) to collate data and facilitate annotation. The DNA and encoded protein sequences of related species were compared using the Artemis Comparison Tool (ACT) (K. Rutherford, unpublished; http://www.sanger.ac.uk/Software/ACT/). Orthologous gene sets were calculated by reciprocal best match FASTA comparisons, with subsequent manual curation. Pseudogenes had one or more mutations that would ablate expression; each of the inactivating mutations was subsequently checked against the original sequencing data.

Genomic comparison of *Bacteroides fragilis* with *Bacteroides thetaiotaomicron*

The recently sequenced *B. thetaiotaomicron* strain VPI-5482 (ATCC 29148)(*6*), has a larger genome of 6.26 Mb containing 4779 predicted genes and an unusually low ratio of gene number to genome size with 0.763 genes/kb (*6*). The proportion of the genome devoted to protein coding (89.3%) is, however, not unusual. This is explained by a high average gene length (1173 bp), and abundant large proteins of over 600 aa. At 1087bp the average gene length in *B. fragilis* is slightly shorter, but it is still amongst the highest so far reported in bacteria.

Despite their con-generic taxonomic status, *B. fragilis* and *B. thetaiotaomicron* share remarkably few orthologous genes (as measured by reciprocal-best-match FASTA analysis). Only 2,384 predicted genes are shared, with *B. fragilis* containing

1890 unique genes (44.3 % of its coding capacity), and *B. thetaiotaomicron* containing 2337 (48.9 %). This is considerably greater than the number of genes unique to *Salmonella* vs. *Escherichia* (*7*), or to *Escherichia* vs. *Yersinia* (*8*) indicating the breadth of diversity amongst *Bacteroides*. This is underlined by the average amino-acid identity between orthologous gene pairs; just 76.6%.

A comparison of *B. thetaiotaomicron* with *B. fragilis* was carried out to determine the nature of the non-orthologous genes. A particularly interesting feature is the extent of diversity of cell envelope, transmembrane, polysaccharide and outer membrane proteins compared to *B. thetaiotaomicron* (Fig. S2); *B. fragilis* unique surface proteins include 128 inner membrane proteins, 2 peptidoglycan associated proteins and 57 predicted outer membrane proteins. In addition, there are 102 unique surface polysaccharide biosynthesis genes, arranged in 10 gene clusters (seven of which are controlled by invertible promoters). This variation in surface polysaccharides and other antigens may be significant in relation to the difference in the pathogenic potential between *B. fragilis* and *B. thetaiotaomicron*. In terms of transcriptional regulators, the most abundant class is the AraC family of transcriptional regulators, with 20 being unique compared to *B. thetaiotaomicron*. An additional point of interest in the *B. thetaiotaomicron* genome is the significantly increased number of sigma factors and anti-sigma factors relative to genome size; of a total of 45 sigma factors in *B. fragilis* there are 16 that are not conserved in *B. thetaiotaomicron*.

Additional DNA inversions

In addition to the restriction/modification intergenic shufflon, a further three different systems were identified. The simplest involves BF0335 and BF0336 (IR-P), which encode two parts of a sensor-regulator system. BF0336 encodes the putative sensor domain and transmembrane domain, and BF0335 encodes the histidine kinase, response receiver and DNA binding domains. The first CDS is flanked by inverted repeats su such that recombination will fuse the two into a single coding sequence. Other fused sensor-regulator genes, lacking invertible regions are present both in *B. fragilis* and *B. thetaiotaomicron* (*6*).

A further two more complex systems involve gene pairs similar to *ragA* (*susC* - like) and *ragB* of *P. gingivalis* (Fig. 1). In *P. gingivalis* RagB is a major immunodominant surface antigen and *ragA/B* positive strains are associated with sites of periodontal destruction (*9*) SusC is an outer membrane protein, which in association with SusD, binds starch at the bacterial surface in *B. thetaiotaomicron* (*10*). In one system, five *ragA/B* like gene pairs (BF1716/8, BF1719/20, BF1722/21, BF1798/7 and BF1803/2; IR-EE), at either end of a ~90 kb region of the chromosome, have an extensive repeat sequence (over 200bp) that overlaps the start codon of the *ragA* homologs (Fig. 1C). One of these (BF1803) is downstream of an invertible promoter (Table S2A, Group 2; IR-T), and recombination between these larger repeats could bring any one of the alternative gene pairs downstream of this promoter by inversion of the intervening sequence. Evidence that this was indeed occurring was found in the shotgun data. A potential recombinase (BF1795) is located close to BF1797/9 and is associated with the putative conjugative transposon encoded within the 90kb central region.

A second independent system involves a further group of four co-located *ragA/B* like gene pairs (BF0590, BF0592, BF0594 and BF0596; Table 1b, IR-CC), where three of the *susC* (*ragA*-like) homologues lack an appropriate start codon. Within the 5' end of the coding region of these genes is a 60bp inverted repeat, identical to that found 68 amino-acids downstream of the start codon of the fourth, BF0594 (Fig. 1C). Recombination between these inverted repeats would fuse the coding sequences of any of these genes to the promoter and translation initiation signals of BF0594. Again, evidence was seen that this was occurring in the shotgun. Another potential recombinase (BF0593) is encoded adjacent to these genes. Examples of similar multiple intergenic inversions include *omp1* of *Dichelobacter* (formerly *Bacteroides*) *nodosus* and the variable surface antigens of *Mycoplasma pulmonis* (*11*). There are also invertible promoter regions upstream of nine other *susC* homologues (Table S2A) and several other predicted outer membrane proteins belonging to a related family (Table S4). This indicates that there is a strong selective pressure in favour of the variation of these molecules, which suggests that they play an important role in the survival of *B. fragilis*.

Several invertible promoters are between divergent coding regions, thus potentially driving variable transcription of other genes. These include a sialoconjugate degradation operon (IR-T; BF1804-BF1817); a sigma factor (IR-W; BF2944), indicating the possibility of cascade regulation; and the *cpn10-cpn60* operon encoding the major chaperones GroES and GroEL (IR-M). These genes are known to be essential, and have not been seen to be phase-variable, in other organisms. Analysis of the promoter region for *cpn10* in *B. thetaiotaomicron* shows that it is

flanked by 29bp inverted repeats whose sequence is similar to that of *B. fragilis*, although it has not been identified as being invertible.

An 11kb region of the plasmid (pBF9343) containing partition, replication and mobilisation functions, and the invertase *finB*, undergoes inversion (Fig. S1B). There appears to be no clear functional consequence of this, although it may affect the transcription of *finB*, as one of the ends of the inversion is ~100 bp upstream of its start site. As both FinB and the chromosomally-encoded FinA (MpiA) may be involved in inversion of *hin*-like invertible promoters, this raises the possibility of a random variation in the rate of variation at those promoters in plasmid-containing strains.

Virulence associated genes

Iron is essential for the growth of most bacteria. Within the human host iron is sequestered such that the free iron concentration is estimated to be 10^{-18} M, well below the concentration necessary to support bacterial growth. To overcome this iron famine, pathogenic bacteria have evolved specific adaptive mechanisms for obtaining iron. One is the production of secreted iron chelating compounds termed siderophores that sequester iron and are subsequently re-imported through membrane receptors (*12*). Internalisation is generally performed by TonB-dependent outer-membrane receptors, of which *B. fragilis* contains at least 57, and is energised by the TonB/ExbBD complex (BF3737, BF3738 and BF3739). An FhuA (*Vibrio cholerae* ferrichrome receptor) homologue, together with a two-component sensor-regulator pair and a complete sigma, anti-sigma and anti-anti-sigma factor system (BF2844, BF2842 and BF2843) is evident. Whether *B. fragilis* synthesises siderophores or

utilizes those produced by other bacteria remains to be determined, although a non-ribosomal peptide synthase (BF2837), an enzyme family known to be involved in the biosynthesis pathway for other siderophores, is located nearby and may be part of a siderophore biosynthesis system. In addition, there are two periplasmic binding-protein dependent iron uptake systems, of the ferric citrate FecCD family (BF1185 and BF2247), each with an associated periplasmic iron-binding protein. The putative siderophore uptake systems appear to be independent of the already identified iron-repressible haem uptake protein HupA that forms part of a haem binding outer membrane protein complex (*13*). Although growth is severely limited in the absence of haem (*14*), *B. fragilis* does not produce zones of haemolysis on blood agar. There are, however, 13 CDSs, fewer than in the *B. thetaiotaomicron* genome, which may encode a haemolytic function. One of these (BF0270) is similar to the haemolysin A of *Prevotella melaninogenica (*formerly *Bacteroides melaninogenicus)*. Interestingly, within the associated CDSs (BF0266-0269) there is a putative haem receptor, suggesting that this might be a haem acquisition operon.

Extracellular enzymes, potentially capable of degrading components of the host's extracellular matrix, host cells and tissue, and therefore potentially involved in *B. fragilis* virulence, include hyaluronidase, chondroitin sulphatase, fibrinolysin, DNAase, lipases, proteases and neuraminidases (*15*). Bacterial sialidases or neuraminidases that remove the sialic acid residues from host oligosaccharides are implicated in bacterial virulence as they potentially interfere with normal host cell function. The neuraminidase NanH (BF1806) has previously been sequenced from *B. fragilis* and a second, highly similar, gene is present (BF4051), which could be equivalent to the second previously reported in *B. fragilis* strain SBT3182 by Tanaka,

et al. (*16*) The observed degradation of hyaluronic acid, a component of the host's extracellular matrix, may be due to BF3796, which is similar to the hyaluronidase encoded by a *Streptococcus pyogenes* bacteriophage (*17*). Three putative tricorn-like proteases (BF0080, 2517 and 3752) with similarity in the beta-propeller, PDZ and catalytic domains are evident, but as in the MdsD protein of *Prevotella* sp, they also contain a putative signal peptide (*18*). Which of the potentially secreted putative 23 peptidases and 2 lipases are involved in virulence rather than nutrition remains to be determined.

Attachment to host tissues is key to the virulence of many pathogenic bacteria, and can be promoted by a variety of adhesins. The non-capsulate/EDL population of *B. fragilis* haemagglutinates erythrocytes (*19*). One haemagglutinin (BF1428) that has a homologue in *B. thetaiotaomicron* is evident; however, no fimbrial genes have been identified, despite reports of their observation in other strains (*20*). There is no evidence for type III, IV, autotransporter or two-partner secretion systems nor flagellar biosynthesis systems in the *B. fragilis* genome, hence secreted virulence determinants are likely to be exported via Hly-type I secretion systems such as BF0010 and BF0011, BF0608 and BF0610 or BF3811 and BF3812, which are similar to the haemolysin type I secretion system HlyDB from *E. coli* (*21*), or via the type II general secretion pathway. *B. fragilis* produces copious quantities of enzyme-containing outer membrane vesicles (*22*). This may therefore be an important export mechanism.

DNA recombination and repair of metronidazole induced DNA damage

The current drug of choice in treating *B. fragilis* infections, metronidazole, is activated intracellularly via anaerobic reduction of the nitro group and interacts with DNA causing strand breaks. RecA-mediated strand exchange is required for repair of DNA breaks, the importance of which is shown by the sensitivity of a *B. thetaiotaomicron recA* mutant to metronidazole and other DNA damaging agents (*23*). RecA (BF1180) is 60% identical to *E. coli* RecA and can complement an *E. coli recA* mutant, demonstrating that DNA repair pathways are conserved in *B. fragilis*(*24*).

Two major pathways of recombination have been extensively studied in the model organisms *E. coli* and *Bacillus subtilis*; single-strand gap repair mediated by the RecFOR proteins and double-strand break repair (reviewed in ref. *25*) Specific DExx motif helicases, belonging to Superfamily I, are essential in cells expressing RecF; these are Rep and UvrD in Gram negative bacteria and PcrA in Gram positive bacteria (*26*). *B. fragilis* contains a total of 24 putative DNA helicases, compared to 11 in *E. coli*, the functional significance of which is unknown. RecFOR homologues are present in *B. fragilis* together with two homologues of the PcrA helicase usually found in Gram positive bacteria. The presence of single PcrA homologues has been suggested to be a hallmark of Gram positive bacteria (*26*). It remains to be determined whether the two homologues in *B. fragilis* have separate roles or overlapping functions analogous to the activities of Rep and UvrD in *E. coli*.

Double-strand break repair is mediated by RecBCD in *E. coli* and AddAB in *B. subtilis* (*25*). *B. fragilis* contains a CDS (BF0679) encoding a homologue of RecD but does not contain identifiable homologues of RecBC or AddAB. The RexAB ATP-dependent exonuclease/helicase has been implicated in repair of double-strand breaks

in the Gram positive bacterium *Lactobacillus lactis* (*27*). There is a single RexA (BF2192) homologue in *B. fragilis* but no identifiable RexB. A potential hypothesis is that double-strand break repair in *B. fragilis* is mediated by a novel mechanism where the joint action of the RexA and RecD helicases unwind the DNA duplex with concurrent degradation by the exonuclease function of RexA until the equivalent of a chi site is encountered. This combination of helicases of opposite polarities (RecD has 5'-3' while RexA has 3'-5' activity) would be analogous to the helicase functions present in the RecBCD complex (*28*).

The sequence and annotation of the genome are available with further details from http://www.sanger.ac.uk/Projects/B_fragilis/.

Supporting Figures:

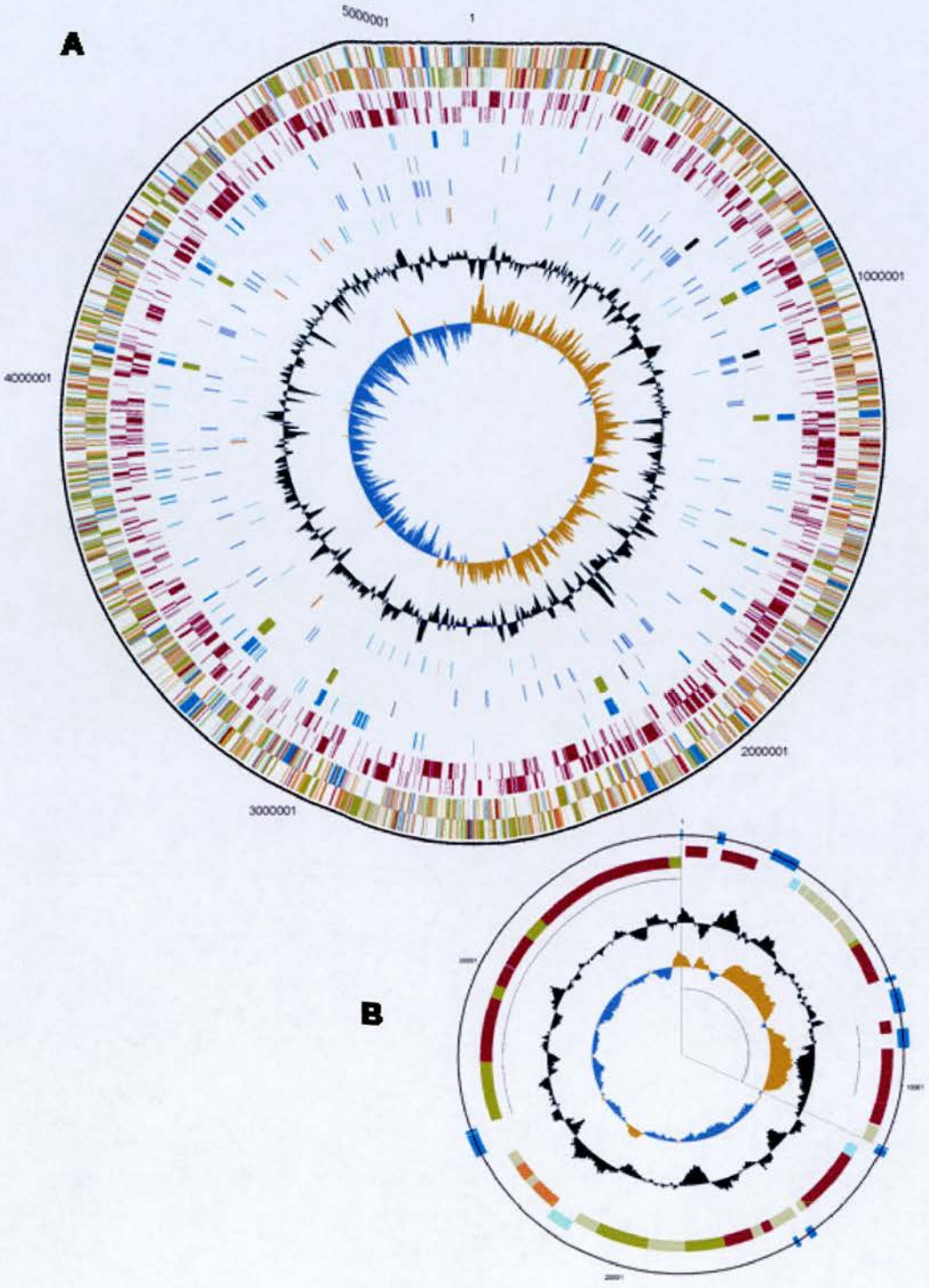

Figure S1: A) Circular representation of the *B. fragilis* NCTC9343 chromosome: From the outer to the inner circle: Circle 1: DNA coordinates (origin in base 1); Circles 2+3: all CDSs

(forward and reverse strands); Circles 4+5: Unique CDSs in *B. fragilis* as compared with *B. thetaiotaomicron* CDSs (forward and reverse strands); Circle 6: Pathogenicity related CDSs (blue); Circle 7: polysaccharide biosynthesis clusters (green) and invertible regions (black); Circle 8: SusC homologues (pink); Circle 9: rRNAs (orange) and tRNAs (blue); Circle 10: G+C content (plotted using a 10Kb window); Circle 11: GC skew ((G-C)/(G+C)) (plotted using a 10Kb window). Colour coding for circles 2 and 3: dark blue; pathogenicity/adaptation, black; energy metabolism, red; information transfer, dark green; surface associated, cyan; degradation of large molecules, magenta; degradation of small molecules, yellow; central/intermediary metabolism, pale green; unknown, pale blue; regulators, orange; conserved hypothetical, brown; pseudogenes, pink; phage+IS elements, grey; miscellaneous. **B)** Circular representation of the pBF9343 plasmid: From the outer to the inner circle: Circle 1: DNA coordinates with repeats marked as blue boxes; Circles 2+3: CDSs (forward and reverse strands); Circle 4: Transfer region marked with black line; Circle 5: Mobilisation region marked with black line; Circle 6: G+C content (plotted using a 1Kb window); Circle 7: GC skew ((G-C)/(G+C)) (plotted using a 1Kb window); Circle 8: Inverted region marked with black line.

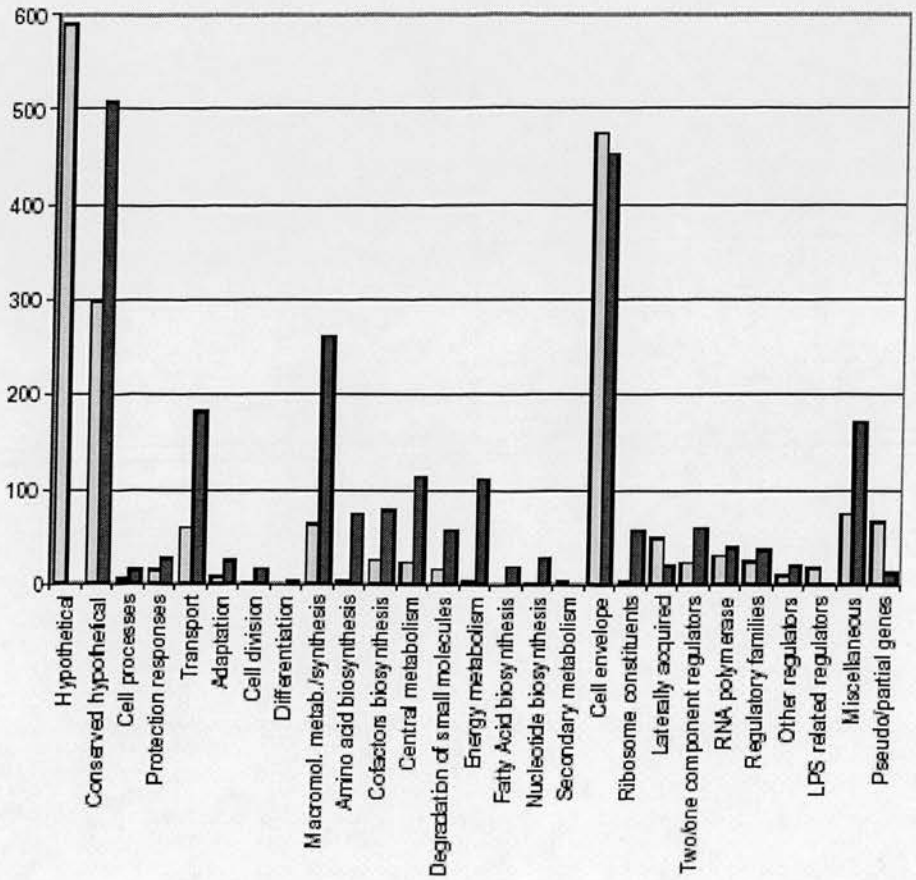

Figure S2: Numbers of orthologous genes between *B. fragilis* and *B. thetaiotaomicron* (dark grey), and genes unique to *B. fragilis* (light grey) within specific functional categories.

Supporting Tables:

Table S1 - General features of the *B. fragilis* genome.

Chromosome	Size (bp)	5,205,140
	G+C content (%)	43.19
	CDSs	4,274
	of which pseudogenes	70
	Coding density (%)	88.1
	Average gene length (bp)	1,091
	Ribosomal RNAs	19
	Transfer RNAs	73
	IS/transposon elements	24
Plasmid pBF9343	Size (bp)	36,560
	G+C content (%)	32.24
	CDSs	48
	Coding density (%)	85.0
	Average gene length (bp)	652

Table S2A - Invertible promoters.

Group	Average length of *fin* regions (bp)	Invertible region (IR)	Coordinates	Active in shotgun	Regulated CDSs
1	226	D	3032390.. 3032595	Yes	PS E polysaccharide biosynthesis region
		DD	894511.. 894721	No	PS G polysaccharide biosynthesis region.
		E	1634575.. 1634805	Yes	PS A polysaccharide biosynthesis region.
		F	4091660.. 4091889	Yes	PS H polysaccharide biosynthesis region.
		G	2211236.. 2211454	Yes	PS B polysaccharide biosynthesis region.
		H	4361354.. 4361586	Yes	PS D polysaccharide biosynthesis region.
		I	1806791.. 1807023	No	PS F polysaccharide biosynthesis region.
	161	A	89789.. 89949	No	Putative membrane protein.
		B	91906.. 92066	Yes	Hypothetical protein. Putative type I restriction-modification enzyme.

		C	129406.. 129566	Yes	Hypothetical protein. Membrane protein. Putative SusC homologue surface membrane protein.
		J	4866094.. 4866254	Yes	Conserved hypothetical protein. Hypothetical protein.
		K	4868436.. 4868596	No	Hypothetical protein. Putative membrane protein.
2	370	AA	603197.. 603530	Yes	Putative exported protein.
		L	5046156.. 5046613	Yes	Putative exported protein. Putative phosphoenolpyruvate carboxykinase.
		M	3790067.. 3790365	Yes	10kDa chaperonin GroES. Putative exported protein.
		N	5023725.. 5024147	Yes	Putative outer membrane protein. Putative pyruvate carboxylase biotin-containing subunit.
		O	2282840.. 2283132	Yes	Putative outer membrane protein.
		T	2093020.. 2094551	Yes	Putative gene cluster for degradation of sialoconjugates.
		V	4218300.. 4218701	No	Putative exported protein. Conserved hypothetical protein. Putative anti-sigma factor.
		W	3420744.. 3421217	No	Putative anti-sigma factor. Putative ECF-sigma factor, RpoE-like.
		X	3754426.. 3754869	No	Putative exported protein. Putative outer membrane protein.
		Y	3839227.. 3839616	Yes	Putative outer membrane protein. Putative outer membrane receptor protein.

		Z	1121569.. 1121954	No	Putative enoyl ACP-reductase.

Table S2B - Other invertible regions.

Invertible Region (IR)	Coordinates	Active in shotgun	CDSs involved	effect
BB (multiple inversions)	2147598.. 2151089	Yes	Putative type I restriction-modification endonuclease specificity subunit. BF1839, BF1840, BF1841, BF1842	Exchange of DNA-binding modules in specificity subunit
CC (multiple inversions)	708692.. 720999	Yes	Putative outer membrane proteins. BF0590, BF0592, BF0594, BF0596	Switching of alternative outer membrane proteins onto translational start signals (fixed promoter)
EE (multiple inversions)	1999949.. 2093942	Partially	Putative outer membrane proteins. BF1716, BF1719, BF1722, BF1798, BF1803	Switching of alternative outer membrane proteins to control by invertible promoter in IR-T
P	401492.. 403339	Yes	Putative two-component sensor histidine kinase/response regulator fusion.	Fusion and separation of sensor and phospho-relay components
Q	2831962.. 2833445	Yes	Hypothetical proteins. BF2439A, BF2439B	Alternative orientation of two hypothetical proteins (with or against direction of transcription of surrounding genes)
R	1073991.. 1079515	Yes	Putative outer membrane proteins. BF0865, BF0866	Alternative orientation of genes (with or against direction of transcription of surrounding genes
S	1079501.. 1087414	Yes	Putative outer membrane proteins. BF0867, BF0868 Putative RNA polymerase ECF-sigma factor and putative anti-sigma factor. BF0869, BF0870	Alternative orientation of genes

Table S3 – Polysaccharide biosynthesis operons.

Region	Coordinates	Variable promoter (IR)	Active in shotgun	Regulators	CDSs
PS A	1635470..1635940	E	Yes	UpaY / UpaZ	BF1367 – BF1377
PS B	2211582..2234865	G	Yes	UpbY / UpbZ	BF1893 – BF1914
PS C	1260915..1277396	Non-variable	-	UpcY / UpcZ	BF1009 – BF1026
PS D	4346276..4361140	H	Yes	UpdY / UpdZ	BF3683 – BF3699
PS E	3016482..3032253	D	Yes	UpeY / UpeZ	BF2591 – BF2606
PS F	1807211..1822424	I	No	UpfY / UpfZ	BF1549 – BF1565
PS G	894922..917651	DD	No	UpgY / UpgZ	BF0731 – BF0752
PS H	4076795..4091435	F	Yes	UphY / UphZ	BF3451 – BF3466
PS I	3249979..3278123	Non-variable	-	UpiY	BF2790 – BF2817
PS J	1987522..2004910	Non-variable	-	-	BF1706 - BF1718

Table S4 – Putative SusC homologues.

CDS	Coordinates	Product	Invertible region
BF0229	250479..253874	Putative SusC homologue outer membrane protein	No
BF0288	334274..337474	Putative SusC homologue TonB-dependent outer membrane protein	No
BF0334	397798..401148	Putative SusC homologue outer membrane protein	No
BF0341	412037..415084	Putative TonB-dependent outer membrane exported protein	No
BF0349	420462..423518	Putative TonB dependent outer membrane exported protein	No
BF0381	465995..469327	Putative exported protein	No
BF0501	581461..584628	Putative outer membrane protein	No
BF0518	603565..606753	Putative outer membrane protein	No
frrG (BF0536)	629760..633170	Putative outer membrane protein	No
BF0571	680148..683426	Putative TonB-linked outer membrane receptor protein	No
BF0578	694072..696408	Putative TonB-dependent outer membrane receptor protein	No

BF0590	705773..708751	Putative SusC homologue surface membrane protein	Yes
BF0592	710759..713614	Putative SusC homologue surface membrane protein	Yes
BF0594	715846..718878	Putative SusC homologue surface membrane protein	Yes
BF0596	720940..723876	Putative SusC homologue surface membrane protein	Yes
BF0661	797339..800476	Putative outer membrane protein	No
BF0681	828289..831702	Putative outer membrane protein	No
BF0711	871405..874152	Putative TonB-dependent outer membrane receptor protein	No
BF0759	924125..927259	Putative outer membrane protein	No
BF0807	989559..992741	Putative outer membrane protein	No
BF0864	1070528..1073572	conserved hypothetical protein	No
BF0866	1075945..1079352	Putative outer membrane protein	No
BF0868	1081426..1084734	Putative TonB-linked outer membrane protein	No
BF0871	1087602..1090943	Putative exported protein	No
BF0890	1111869..1115135	Putative outer membrane receptor protein	No
BF0893	1118124..1121387	Putative outer membrane receptor protein	No
BF0971	1213148..1216468	Putative outer membrane protein	No
BF0977	1222361..1225738	Putative TonB-dependent outer membrane receptor protein	No
BF1204	1463991..1467359	Putative outer membrane protein	No
BF1310	1570137..1573439	Putative outer membrane protein	No
BF1415	1679751..1683143	Putative outer membrane protein	No
BF1512	1771475..1774855	Putative outer membrane protein	No
BF1618	1886174..1889299	Putative outer membrane receptor protein	No
BF1716	1999994..2002084	Putative SusC homologue outer membrane protein	Yes
BF1719	2005661..2008927	Putative SusC homologue outer membrane protein	Yes
BF1722	2012226..2015546	Putative SusC homologue outer membrane protein	Yes
BF1798	2081856..2085086	Putative SusC homologue outer membrane protein	Yes
BF1803	2090601..2093867	Putative SusC homologue outer membrane protein	Yes
BF1804	2094629..2097796	Putative TonB-dependent outer membrane receptor protein	No
BF1816	2119766..2122507	Putative outer membrane protein	No

BF1956	2283190..2286564	Putative outer membrane protein	No
BF1992	2330851..2334225	Putative outer membrane protein	No
BF2044	2387138..2389924	Putative TonB-dependent outer membrane receptor protein	No
BF2084	2437288..2439492	Putative TonB-dependent outer membrane receptor protein	No
BF2195	2557432..2560734	Putative exported protein	No
BF2270	2654934..2658200	Putative exported protein	No
BF2697	3136811..3139111	Putative exported TonB-dependent receptor protein	No
BF2708	3149210..3151351	Putative exported TonB-dependent receptor protein	No
BF2907	3376170..3379502	Putative exported protein	No
BF2942	3416623..3419997	Putative exported protein	No
BF3024	3515789..3518941	Putative exported protein	No
BF3097	3612821..3616255	Putative exported protein	No
BF3146	3670784..3673789	Putative exported protein	No
BF3199	3754969..3758121	Putative exported protein	No
BF3258	3835857..3839033	Putative exported protein	No
BF3307	3899923..3903075	Putative exported protein	No
omp117 (BF3412)	4024110..4027376	Putative outer membrane protein	No
BF3444	4066003..4069050	Putative membrane protein	No
BF3572	4206914..4210357	Putative membrane protein	No
BF3576	4214892..4218104	Putative exported protein	No
BF3581	4222187..4225660	Putative membrane protein	No
BF3642	4297965..4300640	Putative exported protein	No
BF3712	4374951..4378244	Putative exported protein	No
BF3724	4392109..4395414	Putative exported protein	No
BF3746	4419207..4422644	Putative membrane protein	No
BF4056	4769105..4772347	Putative outer membrane protein	No
BF4062	4781654..4784647	Putative TonB-linked outer membrane protein	No
BF4132	4860945..4864271	Putative outer membrane protein	No
BF4169	4911410..4914403	Putative TonB-linked outer membrane protein	No
BF4178	4932416..4935583	Putative outer membrane protein	No
BF4246	5030783..5033878	Putative outer membrane protein	No

BF4248	5030783..5033878	Putative outer membrane protein	No
BF4256	5046824..5050081	Putative outer membrane protein	No
BF4268	5067453..5070962	Putative outer membrane protein (pseudogene)	No
BF4323	5135539..5138736	Putative TonB-dependent outer membrane receptor protein	No

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Non-Sporing Gram-Negative Anaerobes

Sheila Patrick[1] and Brian I. Duerden[2]

[1]Department of Microbiology and Immunobiology, School of Medicine, Queen's University of Belfast, Belfast; and [2]Anaerobe Reference Laboratory, Department of Medical Microbiology, University of Wales College of Medicine, and National Public Health Service for Wales, Heath Park, Cardiff, UK

INTRODUCTION

The major Gram-negative non-sporing anaerobic bacteria of medical interest are classified within the families Bacteroidaceae and Fusobacteriaceae. They are a major component of the normal microbiota of mucosal surfaces of man and the whole range of animals from invertebrates such as termites to primates and are also important pathogens, usually in infections related to those colonised mucosal sites. Their contribution to human infection was first recognised in a report of pulmonary gangrene and appendicitis by Veillon and Zuber from the Institute Pasteur in 1898 although organisms now classified as *Fusobacterium necrophorum* had already been shown to cause calf 'diphtheria' (by Loeffler in 1884), liver abscesses in cattle and facial necrosis in rabbits. In 1861, Pasteur himself had introduced the term anaerobe for microbes that would grow only in the absence of free oxygen. Before 1900, the role of non-sporing anaerobes in suppurative diseases of the middle ear, mastoid and sinuses, pulmonary gangrene and genital tract infection was established by the group of researchers at the Faculté de Medicin de Paris. However, with a few notable exceptions, non-sporing anaerobes were largely ignored in mainstream medical microbiology for most of the 20th century, until the 'anaerobic renaissance' of the 1970s when improved laboratory methods for reliable routine anaerobic microbiology became more widely available. The findings of the early workers were then rediscovered, and the widespread role of these organisms in infections of the abdomen, perineum and genitalia, mouth and respiratory tract, and compromised soft tissues at other sites became more generally recognised (Finegold, 1994).

DESCRIPTION OF THE ORGANISMS

Development of Taxonomy

Veillon and Zuber named their isolates *Bacillus fragilis*, *Bacillus fusiformis* and others, and the current classification dates from 1919 when Castellani and Chalmers proposed the name *Bacteroides* for all obligately anaerobic, non-sporing bacilli; this definition was modified to exclude Gram-positive organisms by Weiss and Rettger in 1937. Knorr introduced the name *Fusobacterium* in 1922 for the spindle-shaped Gram-negative anaerobes. Despite various subsequent proposals for new genus names, all of which were subsequently deemed to be invalid, these two genera were the basis of the classification presented in the eighth (1974) edition of Bergey's Manual of Determinative Bacteriology, with the addition of the single-species genus *Leptotrichia* (*L. buccalis*) for fusiform organisms that produce lactic acid rather than butyric acid as their major metabolic product (Holdeman and Moore, 1974). The genus *Bacteroides* contained five groups of species: 1. *B. fragilis*, divided into five sub-species; 2. bile-sensitive non-pigmented species such as *B. oralis*; 3. *B. melaninogenicus* including all strains that produced black- or brown-pigmented colonies on lysed blood agar and divided into three sub-species; 4. non-saccharolytic non-pigmented species; and 5. other, unrelated saccharolytic species. This was the starting point for the rapid growth of clinical and taxonomic studies with Gram-negative anaerobes (Duerden, 1983).

It should be remembered that although these bacteria are Gram negative, they are not closely related taxonomically to facultative and aerobic Gram-negative bacteria generally encountered in infections such as the Enterobacteriaceae and the pseudomonads. *Bacteroides* and the related *Porphyromonas* and *Prevotella* spp. belong to the Bacteroidetes/Chlorobi superphylum. This group diverged early in evolutionary terms from other eubacteria, and this is thought to have occurred well before the divergence of the Gram-positive bacteria from the phylum that contains Gram-negative species such as *Escherichia coli*, the Proteobacteria. Similarly, the fusobacteria are within their own separate phylum and have similarities at the genome sequence level with low mol% G+C Gram-positive bacteria.

The early taxonomic studies on these bacteria were based upon conventional cultural and biochemical tests, which were difficult to perform reliably and to interpret in anaerobic growth conditions. The use of gas–liquid chromatography (GLC), introduced into this area of work in the late 1960s, was a significant advance in showing the possession of distinct metabolic pathways giving reproducible patterns of fatty acid end products. A recent description of GLC methodology for anaerobes is detailed in the Wadswoth-KTL anaerobic bacteriology manual (Jousimies-Somer *et al.*, 2002). However, the significant advances since 1980 have been based upon a combination of chemotaxonomy and genetic analysis in particular, for example, phylogenetic 16S rRNA gene sequencing. rRNA gene sequencing is now a standard method for the validation of culture identity, and suitable methodology is detailed in, for example, Stubbs *et al.* (2000). A comprehensive taxonomic listing of old and new nomenclature can be found in Jousimies-Somer and Summanen (2002). The reader is directed to the following publications for detailed information on taxonomic history and recent updates (Shah and Garbia, 1991; Jousimies-Somer, 1997; Shah, Gharbia and Duerden, 1998; Jousimies-Somer and Summanen, 2002). The chemotaxonomic characters of the main genera are summarized in Table 45.1.

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Table 45.1 Chemotaxonomic characters of the main genera of Gram-negative anaerobes of clinical importance

Character	*Bacteroides*	*Bilophila*	*Prevotella*	*Porphyromonas*	*Fusobacterium*	*B. ureolyticus*
Major products	Acetate, succinate	Acetate succinate	Acetate succinate	Butyrate	Butyrate	Acetate, propionate
Dehydrogenases	G-6-PDH, 6-PDGH, MDH, GDH	...	MDH, GDH	MDH, GDH	GDA	...
DNA G+C (mol%)	40–48	39–40	40–50	48–54	27–32	28
Peptidoglycan diamino acid	*meso*-DAP	...	DAP	DAP, lysine	DAP, lanthionine	...
Fatty acids	Straight saturated ante*iso*- and *iso*-methyl branched		Straight saturated ante*iso*- and *iso*-methyl branched	*iso*-methyl branched	Straight chain, monounsaturated	...
Quinones	MK-10, MK-11		MK-10, MK-11, MK-13	MK-9	–	...
Sphingolipids	+	...	–	–	–	–
Urease	–	+	–	–	–	+

6-PGDH, 6-phosphogluconate dehydrogenase; DAP, diaminopimelic acid; G-6-PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase.

Bacteroides

The sub-species of *B. fragilis* were given species status, and several new species were added to this fragilis group of intestinal *Bacteroides*. As other species have been removed to new genera, the genus *Bacteroides sensu stricto* should now encompass only those species of the fragilis group. It was also recognised that a single species *B. melaninogenicus*, which had been described by Oliver and Wherry in 1921, could not include organisms as diverse as the saccharolytic subsp. *intermedius* and subsp. *melaninogenicus* and the non-saccharolytic but highly proteolytic subsp. *asaccharolyticus*. *Bacteroides asaccharolyticus* was first separated as a distinct species and then assigned to a new genus, *Porphyromonas*, together with related isolates from diseases of the mouth, *P. gingivalis* and *P. endodontalis*; further new species of human and animal origin were added subsequently. The saccharolytic pigmented species were shown to share many properties with the group 2 non-pigmented organisms that were mostly of oral or genital tract origin. These became the 'melaninogenicus-oralis group' and were then assigned to a new genus as *Prevotella* spp. The other '*Bacteroides*' species that do not belong to these three main genera of human isolates have mostly been assigned to various new genera although a few remain with the inappropriate name '*Bacteroides*' while awaiting reclassification. With the exception of *'B.' ureolyticus* and *Bilophila wadsworthia*, these are of little concern in medical microbiology.

Fusobacterium

The genus *Fusobacterium* is now defined in biochemical and genetic terms as encompassing Gram-negative non-sporing anaerobes with DNA G+C content of 27–33 mol% that produce butyric and propionic acids as major metabolic products. Many that conform with this description do not exhibit the classical fusiform appearance. The more significant members of the genus in human disease are *F. necrophorum* and *F. nucleatum*.

General Properties

All members of the families Bacteroidaceae and Fusobacteriaceae are obligately anaerobic, Gram-negative bacilli, although many are highly pleomorphic and microscopic appearances range from cocco-bacillary to long filamentous forms. Their susceptibility to oxygen varies; some, such as *B. fragilis*, can remain viable (sub-cultivable) after exposure to oxygen for many hours after growth on agar media, whereas some others cannot withstand exposure for more than a few minutes. Most of those of concern in medical microbiology are at the more tolerant end of this spectrum and can be manipulated on the open bench, although the speed of regrowth and percentage viability are generally better if bacteria are handled in an anaerobic cabinet without exposure to air. Anaerobic conditions for growth are provided by incubation in anaerobic jars, or cabinets or other small-scale culture systems that exclude air and have a chemical system for removing traces of oxygen. The most commonly used gas mixture is H_2 10%, CO_2 10%, N_2 80% for cabinets, or jars that incorporate a palladium catalyst to ensure the removal of remaining oxygen by reaction with the hydrogen. The species assigned to the main genera of are listed in Table 45.2. The genera *Bacteroides*, *Prevotella* and *Porphyromonas* all fall taxonomically within the class Bacteroides, whereas the genera *Fusobacterium* and *Leptotrichia* are within the class Fusobacteria. Details of the taxonomic status and classification of bacteria, including anaerobes, can be found at the NCBI taxonomy web site http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/.

Bacteroides

Bacteroides fragilis and other members of the genus *Bacteroides sensu stricto* are indistinguishable by microscopy or by colonial appearance. They are small, moderately pleomorphic, non-motile Gram-negative bacilli; cocco-bacilli are common but long filaments

Table 45.2 Gram-negative anaerobic bacteria of clinical significance

Bacteroides	*Prevotella*	*Porphyromonas*	*Fusobacterium*
B. fragilis	*Pr. melaninogenica**	*P. asaccharolytica*	*F. necrophorum*
B. distasonis	*Pr. denticola**	*P. gingivalis*	*F. nucleatum*
B. ovatus	*Pr. loescheii**	*P. endodontalis*	*F. varium*
B. thetaiotaomicron	*Pr. intermedia**	*P. canontiae***	*F. pseudonecrophorum*
B. vulgatus	*Pr. nigrescens**		*F. naviforme*
B. uniformis	*Pr. corporis**	*P. macacae*	*F. mortiferum*
B. eggerthii	*Pr. tannerae**	*P. levii*	*F. russii*
B. caccae	*Pr. pallens**	*P. gulae*	*F. ulcerans*
B. merdae			
B. stercoris	*Pr. buccae*		
B. capillosus	*Pr. oris*		
B. coagulans	*Pr. buccalis*		
B. putredinis	*Pr. oralis*		
	Pr. dentalis		
	Pr. enoeca		
(*'B.' splanchnicus*)	*Pr. veroralis*		
	Pr. heparinolytica		
...	*Pr. oulora*		
	Pr. zoogleoformans		
Bilophila wadsworthia	*Pr. bivia*		
	Pr. disiens		
...			
'B.' ureolyticus			

*Black- or brown-pigmented *Prevotella* spp.
**Non-pigmented *Porphyromonas* spp.

are rare. They grow well on conventional laboratory media containing blood to form circular, low convex, smooth, shiny, translucent or semi-opaque, grey colonies, 1–3 mm in diameter, that are often moist or frankly mucoid after incubation for 24–28 h. Most strains are non-haemolytic, but a few are slightly haemolytic and a small proportion are distinctly β-haemolytic. They are strongly saccharolytic organisms, producing acid from a range of carbohydrates, and moderately proteolytic. The main end products of metabolism detected using GLC are acetic and succinic acids, with smaller amounts of various other acids but not *n*-butyric or lactic acids. Growth is generally stimulated by whole bile but inhibited by sodium deoxycholate and isolates are tolerant of the bile salt sodium taurocholate. *Bacteroides splanchnicus* is an intestinal *Bacteroides* that differs from the other true *Bacteroides* spp. in producing propionic and *n*-butyric acids and in genetic and chemotaxonomic characters; it probably represents a distinct genus.

Prevotella

The various species of *Prevotella* cannot be distinguished by cell morphology. Most are short pleomorphic Gram-negative bacilli, often with many cocco-bacillary forms. They are divided into pigmented and non-pigmented groups by the ability of some to assimilate haemoglobin from blood in the medium, converting it to protohaemin which accumulates in the cells, making the colonies dark brown or black after incubation for several days. Most pigmented strains are haemolytic, and pigmentation depends upon lysis of the red cells in the medium and develops most rapidly on lysed blood agar. Pigmentation can vary from almost black to pale brown. Colonies of the non-pigmented species are indistinguishable from each other and very similar to those of the *Bacteroides* spp. – 1–2 mm in diameter, circular, shiny, convex and light buff or grey coloured; most are non-haemolytic. *Prevotella* spp. are moderately to strongly saccharolytic. All produce acid from glucose and various other carbohydrates and, like *Bacteroides* spp., their major metabolic products are acetic and succinic acids; *n*-butyric acid is not produced. Most species are moderately to strongly proteolytic. They are inhibited by bile and bile salts.

Porphyromonas

Formerly known as the asaccharolytic *B. melaninogenicus* strains, *Porphyromonas* spp. are similar to pigmented *Prevotella* spp. in microscopic and colony morphology. They are small, mainly cocco-bacillary organisms with few long filaments. Growth is slower than with *Bacteroides* spp. and some *Prevotella* spp.; small colonies may be visible after incubation for 24 h but many do not appear until 48 h when they are <1 mm in diameter, smooth, shiny and grey. Dark brown or black pigmentation develops after 3–7 days on blood agar, more rapidly on lysed blood agar. After 4–5 days, they are 1–2 mm in diameter and haemolytic – lysis of the cells being essential for pigmentation. Most strains have a characteristically strong, putrid smell, even more noticeable than those of other non-sporing anaerobes. They do not produce acid from glucose or other carbohydrates but their metabolism, as with all anaerobes, is fermentative. The major metabolic end product is *n*-butyric acid. Like *Prevotella* spp., *Porphyromonas* spp. are inhibited by bile or bile salts. They are vigorously proteolytic, and this is considered to be a factor in their virulence.

Fusobacterium

Fusobacteria are anaerobic Gram-negative bacilli of varied size and morphology. Classical fusobacteria, such as *F. nucleatum* are fairly long (4–6 μm), regular bacilli with tapered or pointed ends. Spheroplasts and spindle forms are common. Others (e.g. some strains of *F. necrophorum*) are pleomorphic with a mixture of long, filamentous organisms with rounded ends and many short or even cocco-bacillary forms and numerous spheroplasts and L-forms. Yet others are mostly cocco-bacillary. Colonial appearance is also varied, but most fusobacteria produce moderate to large colonies, 1–3 mm in diameter, generally with an irregular or dentate edge. They vary from translucent to granular and opaque. Their metabolism varies; carbohydrates are fermented only feebly or not at all but most species are proteolytic and the more virulent species such as *F. necrophorum* produce a range of enzymes that can break down tissue components (see p. 536 Tissue damage line 50–62). The major metabolic products of fusobacteria, which help define the genus, are *n*-butyric and propionic acids, the latter produced by deamination of threonine. The G+C content of fusobacteria is low at 27–33 mol%, and these and *Leptotrichia* are phylogenetically distinct from the Bacteroidaceae.

Leptotrichia

The species *L. buccalis* may be synonymous with the original fusiform organism described by Vincent. It has typical, long fusobacterial cells, 5–15 μm long, many with pointed ends. Its colonies are lobate or convoluted, opaque and grey/yellow in colour. Although it is within the Fusobacteriaceae, it differs from *Fusobacterium* spp. in that it produces acid from several sugars and the only major metabolic product is lactic acid, with a small amount of acetic acid; *n*-butyric acid is not produced. Complete 16S rDNA sequencing and DNA-DNA hybridisation of strains initially assigned to *L. buccalis* reveals sufficient genetic diversity to warrant the designation of five different species (including *L. buccalis*) within this group (Eribe *et al.*, 2004).

Bilophila

The single species *Bil. wadsworthia* is a slow-growing, small Gram-negative bacillus that is stimulated by bile, requires pyruvate for growth and is asaccharolytic and urease positive (Baron *et al.*, 1992). It has no genetic homology with *Bacteroides* spp. and is classified within the Desulfovibrionaceae in the phylum Proteobacteria (Laue, Denger and Cook, 1997).

'Bacteroides' ureolyticus

This slender Gram-negative anaerobe typically forms pitting colonies on agar media (hence its former name *B. corrodens*). It is asaccharolytic, strongly proteolytic and urease positive. Its G+C content of 28 mol% clearly sets it apart from the other genera of Bacteroidaceae. Genotypically it is related to the Campylobacteraceae, in the phylum Proteobacteria (Jousimies-Somer, 1997). It is likely that it will be renamed once further similar bacteria have been isolated and studied.

Growth Conditions

As well as requiring anaerobic conditions, most Gram-negative anaerobic bacilli require enriched media for growth; none grows readily on minimal media or unsupplemented nutrient agar. Most require a source of haemin for synthesis of the menaquinones that are essential components of their anaerobic electron transport systems. Some *Prevotella* and *Porphyromonas* spp. also require menadione (vitamin K). Media for optimal growth comprise a rich nutrient base, e.g. Fastidious Anaerobe Agar (Lab M), Columbia agar base (Oxoid), Brucella agar or Wilkins Chalgren agar, with 5–10% blood plus haemin and menadione. *Bacteroides* spp. and some of the more vigorous *Prevotella* spp. produce reasonable growth after 24 h, but other species grow more slowly and require undisturbed anaerobic incubation for 48–72 h, or even longer, for recognisable growth. Details of the growth conditions for anaerobes of clinical importance can be found in the following references (Moore and Moore, 1977; Summanen *et al.*, 1993; Jousimies-Somer *et al.*, 2002).

NORMAL HUMAN MICROBIOTA

All the Bacteroides and Fusobacteria associated with man are part of the normal resident microbiota of the gastrointestinal, oral or genitourinary mucosae where they form a major part of the complex and interdependent ecosystems. However, the species found at the three sites are quite distinct and each species appears to have a distinct ecological niche (Drasar and Duerden, 1991).

Gastrointestinal Tract

This is the normal habitat of all the species of *Bacteroides sensu stricto* (*B. fragilis* group). They are one of the predominant groups of organisms in the faecal and colonic microbiota, having populations of approximately 10^{12} cfu/g wet weight of faeces and outnumbering the facultative enterobacteria by at least 1000:1. Not all species are equally common. The main components of the normal faecal microbiota are *B. vulgatus*, *B. distasonis*, *B. thetaiotaomicron* and *B. uniformis*. Within the gut, *Bacteroides* spp. contribute to the degradation of ingested material, in particular heterologous polysaccharides in plant material, that the mammalian system is incapable of degrading. This process results in the production of short-chain fatty acids, which once absorbed from the gut are potential substrates for energy metabolism in the intestinal mucosal epithelium. The potential for intimate interaction between the normal microbiota and the human host is illustrated by *B. thetaiotaomicron*. Within the environment of the small intestine, *B. thetaiotaomicron* appears to induce villus cells to fucosylate their cell surface glycoconjugates. *Bacteroides thetaiotaomicron* also secretes an α-fucosidase which cleaves the fucose from the villus cell glycoconjugates, thus releasing the fucose, which then becomes a potential carbon and energy source for the bacterium (Hooper *et al.*, 1999). Studies of the adherent mucosal microbiota indicate considerable variation amongst individuals. Mucosal biopsies of the proximal colon and rectum of 12 individuals showed that in different people one or two species of *Bacteroides* predominated by culture (Poxton *et al.*, 1997). For example, *B. fragilis* was the major isolate in one individual, the second most common isolate in three individuals and not detected in six, whereas *B. vulgatus* was the most common isolate in four individuals. Although *B. fragilis* accounts for fewer than 10% of faecal isolates and was not detected in some of the biopsy patients, it is the principal pathogen in gut-associated sepsis (approximately 75% of isolates from abdominal infections). This indicates that *B. fragilis* has a greater pathogenic potential than the other *Bacteroides* spp. *Bil. wadsworthia* is also a common but not dominant member of the faecal microbiota. The gastrointestinal tract is probably also the normal habitat of *P. asaccharolytica* and *'B.' ureolyticus*, but these are present only in small numbers.

Genitourinary Tract

Gram-negative anaerobes are part of the normal microbiota of the vagina although present in relatively small numbers (approximately 10^6 cfu/g of secretions) and outnumbered by Gram-positive species such as lactobacilli. Most isolates belong to the genus *Prevotella*, principally *Pr. bivia* and *Pr. disiens* which are uncommon elsewhere in the body; the pigmented species *Pr. melaninogenica* is also present, but in smaller numbers and less frequently (Drasar and Duerden, 1991).

Mouth

The oral microbiota have long been recognised as a source of a wide range of Gram-negative anaerobic species. The main habitat for these organisms in the mouth is the gingival crevice where both *Prevotella* and *Fusobacterium* spp. make a major contribution to the complex microbiota. The most commonly reported isolates are *Pr. melaninogenica*, *Pr. oralis* and other non-pigmented *Prevotella* spp. and fusobacteria, mainly *F. nucleatum*. *Prevotella intermedia* is also isolated from the normal gingival microbiota but, like *B. fragilis* in the colon, represents a much smaller proportion of the total count of *Prevotella* spp. than do other species. *Porphyromonas gingivalis* is rarely isolated from healthy gingivae (Drasar and Duerden, 1991).

PATHOGENICITY

A wide range of Bacteroidaceae and Fusobacteriaceae have been isolated at one time or another from infections of man but a high proportion of these infections are caused by only a few, more virulent species. Three main features are common to most of these infections: 1. the source of infection is the endogenous microbiota of the patient's own gastrointestinal, oropharyngeal or genitourinary mucosa; 2. alterations of the host tissue, e.g. trauma and/or hypoxia, provide suitable conditions for the development of secondary opportunist anaerobic infections; and 3. the infections are generally polymicrobial, often involving mixtures of several anaerobic and facultative species acting synergically to cause damage. The initiation of infection generally depends on host factors, but even in such opportunist situations, some species show particular pathogenic potential not evident for the majority of related species from the same normal habitat. Thus, most species have some pathogenic capability but the majority of serious anaerobic infections are caused by the small number of more virulent species (Duerden, 1994).

The types of infection generally associated with Gram-negative anaerobes are listed in Table 45.3. The species most commonly isolated and considered to be significant pathogens in these situations are: *B. fragilis* in abdominal infections; *Pr. intermedia*, *P. gingivalis*, and *F. nucleatum* in periodontal disease and other infections related to the mouth; *P. asaccharolytica* and *'B'. ureolyticus* in superficial necrotising infections; and *F. necrophorum* in the invasive disease necrobacillosis (Lemmiere's disease). *Fusobacterium necrophorum* (including subsp. *necrophorum*, formerly Biovar A, and subsp. *funduliforme*, formerly Biovar B) has more of the characteristics of a virulent primary pathogen than other anaerobic species; typically, it causes severe purulent tonsillitis with pseudomembrane formation and lymph node involvement, septicaemia and metastatic abscess formation. It may be fatal even for a previously healthy person.

To establish an infection, bacteria must attach to target cells (generally mucosal or epithelial cells), invade the tissues, establish themselves by multiplying at the site of infection and avoiding elimination by the

Table 45.3 Gram-negative anaerobic bacteria isolated from the normal microbiota and from infections

Normal microbiota	Infections
Faeces	Abdominal
B. vulgatus	*B. fragilis*
B. distasonis	*Bil. wadsworthia*
B. thetaiotaomicron	*B. thetaiotaomicron*
B. uniformis	
Vagina	Genito-urinary
Pr. disiens	*B. fragilis*
Pr. bivia	*Prevotella spp.*
Pr. melaninogenica	*P. asaccharolytica*
	'B'. ureolyticus
Mouth	Head and neck
Pr. melaninogenica	*P. gingivalis*
Pr. intermedia	*Pr. intermedia*
Pr. ovalis	*F. nucleatum*
F. nucleatum	*F. necrophorum*
	Superficial
	P. asaccharolytica
	'B'. ureolyticus
	B. fragilis
	F. ulcerans

host's defence mechanisms and cause damage both to local tissues and, in systemic infections, to the whole patient. A factor that may impact on each of these events is within-strain surface variation in the form of phase and antigenic variation. This type of variation is mediated by genomic rearrangements and is reversible within a single strain (Patrick and Larkin, 1995). Multiple variants may arise during the progression of infection of a single individual or indeed during one subculture in the laboratory. The more virulent Gram-negative anaerobes exhibit virulence factors that help elicit the different stages of infection. A review of the major potential virulence factors of *Bacteroides fragilis* can be found in Patrick (2002). Whole-genome sequencing programmes have added considerably to our knowledge and understanding of pathogenic bacteria and will continue to do so in years to come. Gram-negative non-sporing anaerobe whole-genome sequencing programmes are listed in Table 45.4.

Surface Variation

A single strain of *B. fragilis* may have the capability to reversibly produce three different encapsulating surface structures, namely a large capsule or small capsule, both visible by light microscopy, or an electron-dense layer (EDL) outwith the outer membrane, visible by electron microscopy. In addition, polysaccharides associated with the EDL and large capsulate bacteria (but which do not appear to form the bulk of the large capsule structure) exhibit within-strain antigenic variation (Lutton *et al.*, 1991). This is mediated, at least in part, by invertible promoter regions upstream of the polysaccharide biosynthesis operons. The invertible regions are bound by inverted repeats of 30 or 32 bp in length with striking similarity to the *Salmonella typhimurium* **H** flagellar antigen inversion **cross**-over (*hix*) recombination sites of the invertible hin region (Patrick *et al.*, 2003). In *B. fragilis* NCTC 9343, there are ten different polysaccharide biosynthesis operons. The genes within these operons indicate that some may be similar to enterobacterial 'O-antigen' rather than classical capsules. Analysis of the entire genome has revealed a further 16 regions with similar sequence characteristics, suggesting that these may also be active invertible promoters. Most of these are upstream of conserved putative secreted and outer membrane proteins of unknown function. Also there is evidence of large scale (5–120 kb) DNA inversion events within the genome (Cerdeno-Tarraga *et al.*, 2005).

Whether this phenomenal variation relates to the increased frequency of association of *B. fragilis* with infection when compared with other gastrointestinal tract *Bacteroides* or survival in the gastrointestinal tract remains to be determined.

Adhesion

Bacteroides fragilis can attach to both host cells and components of the extracellular matrix; however, studies of adhesion have rarely taken into account the considerable within-strain surface variation p. 535 Surface Variation line 19–41. Haemagglutination and attachment to host cells in *B. fragilis* is demonstrable in EDL-enriched populations (Patrick *et al.*, 1988). Sodium periodate treatment of the bacteria abolishes haemagglutination, which suggests that saccharides are involved. Populations of strain NCTC 9343 enriched for either a large or small capsule do not haemagglutinate (Patrick *et al.*, 1996). As recent clinical isolates vary considerably in the proportion of bacteria that express the different capsules, this probably accounts for the variation in adhesion observed with different strains. Furthermore, the EDL/non-capsulate bacteria release extracellular vesicles which by themselves will cause haemagglutination. Despite reports of fimbrial expression by *B. fragilis*, fimbrial genes have not been reliably identified, although a gene with similarity to a putative non-fimbrial adhesin described in *Pr. intermedia* is present in *B. fragilis* NCTC 9343 (Cerdeno-Tarraga *et al.*, 2005). Studies of the attachment to components of the extracellular matrix have generally not taken into account the within-strain variability and the potential attachment of bacterial extracellular components; however, there is clear evidence that *Bacteroides* spp. can attach to some host components. Examples include fibronectin, collagen type 1 and vitronectin.

Periodontal pathogens also exhibit adhesive properties. *Fusobacterium nucleatum*, *P. gingivalis* and *Pr. intermedia* adhere to crevicular epithelium and cause haemagglutination. It seems likely that, as with *B. fragilis*, both proteinaceous haemagglutinins and surface saccharides are involved. More than ten genes encoding haemagglutinin-related proteins have been identified in the *P. gingivalis* genome sequence (Nelson *et al.*, 2003). Another aspect of adhesion that may be important in polymicrobial infections is the ability of bacterial species to coaggregate. These coaggregates may be important in creating appropriate conditions for the symbiotic metabolism and pathogenic interaction characteristic of anaerobic infections. This feature is manifest by oral *Prevotella* and *Porphyromonas* spp. together with streptococci, actinomyces and other oral species.

Fusobacterium necrophorum haemagglutinates erythrocytes from a range of species, including humans, and some will also aggregate human platelets. It is suggested that this causes intravascular coagulation which contributes to the establishment of infection (Hagelskjaer Kristensen and Prag, 2000).

Invasion

Most anaerobic pathogens are not primarily invasive. Initiation of infection depends on initial damage due to trauma, hypoxia, neoplasia or some other alteration to provide the route of entry for the anaerobes. *Fusobacterium necrophorum* is an exception to this rule, as although it may be present as part of the normal microbiota, in some patients it penetrates the mucosa. The reasons for this are unknown but may relate to underlying bacterial or viral pharyngitis leading to a reduction in the host defence at the mucosal surface (Hagelskjaer Kristensen and Prag, 2000).

Table 45.4 Complete genome sequencing projects of Gram-negative anaerobes of clinical importance

Species	Strain	Genome size (Mb)	Reference
Bacteroides fragilis	NCTC 9343	5.21	Cerdeno-Tarraga *et al.* (2005)
	638R	5.44	In progress, The Wellcome Trust Sanger Institute *http://www.sanger.ac.uk*_NCBI Accession No. NC_003228
	YCH 46	5.27	Kuwahara *et al.* (2004)
Bacteroides thetaiotaomicron	VPI-5482	6.2	Xu *et al.* (2003)
Fusobacterium nucleatum subsp. *vincentii*	ATCC 49256	2.12	Kapatral *et al.* (2003)
Fusobacterium nucleatum subsp. *nucleatum*	ATCC 25586	2.17	Kapatral *et al.* (2002)
Porphyromonas gingivalis	W83	2.34	Nelson *et al.* (2003)
Prevotella intermedia	17	3.33	In progress, The Institute for Genomic Research *http://www.tigr.org*_NCBI Accession No. NC_003441
Tannerella forsythensis	ATCC 43037	Unfinished	In progress, The Institute for Genomic Research *http://www.tigr.org*_NCBI Accession No. NC_003915

Establishing Infection

Once the initial damage has allowed the anaerobes to penetrate the tissues, they must establish a focus of infection by multiplying and avoiding elimination by the host's defence mechanisms. Most anaerobic infections are polymicrobial, and the metabolic interdependency of the bacterial mixtures involved (both anaerobic and facultative species) is important to their establishment in the tissues, the satisfaction of their nutritional requirements and the expression of their synergic pathogenicity (e.g. Rotstein, Kao and Houston, 1989); thus, the virulence of anaerobic species is a reflection of their ability to exploit a compromised host environment. Tissue damage and necrosis, a reduction in blood supply leading to hypoxia and the presence of a blood clot or foreign body or substance (especially $CaCl_2$) create conditions appropriate for anaerobic growth. The capacity of facultative species such as *E. coli* to consume oxygen may help create reduced conditions favourable to the growth of anaerobes. *Bacteroides fragilis* has a cluster of genes, the aerotolerance operon, whose products aid its survival on exposure to oxygen. A similar cluster had been identified in the *P. gingivalis* genome. In addition, *B. fragilis* can grow in the presence of nanomolar concentrations of oxygen and this is linked to the presence of a cytochrome *bd* oxidase (Baughn and Malamy, 2004).

Gram-negative anaerobes require several growth factors and nutrients produced by damaged host tissues or by other bacteria acting in synergy. For example, *B. fragilis* uses haemoglobin and haemaglobin–haptoglobin complexes as sources of iron and the porphyrin component. It has an iron-repressible haem-binding outer membrane protein involved in the uptake of this essential growth factor (Otto *et al.*, 1996). *Porphyromonas gingivalis* demetalates protohaem to protophorphyrin, providing for its iron needs. Novel putative haemolysin genes with sequence similarity have been identified in *B. fragilis*, *Pr. melaninogenica*, *Pr. intermedia* and *P. gingivalis*. Hydrolytic enzymes and proteases produced by *P. gingivalis* (e.g. gingipains), *P. asaccharolytica*, *Pr. intermedia* and *Pr. denticola* release nutrients from growth factors for these species themselves and for other members of the polymicrobial ecosystems. Some of the proteases of *P. gingivalis* are subject to the post-translational addition of oligosaccharides within the catalytic domain (Gallagher *et al.*, 2003). *Bacteroides fragilis* produces a serine-thiol-like protease that hydrolyses the α-chain of fibrinogen (Chen *et al.*, 1995). As soluble fibrinogen is converted to insoluble fibrin as part of the normal blood-clot formation and wound healing, this enzyme has the potential to slow clot formation. It also produces heparinase and condroitin sulphatase, which hydrolyse heparin and condroitin sulphate, allowing their use as nutrients. The activities of these enzymes are likely to encompass tissue destruction and, where cells and molecules of the immune system are attacked, evasion of the immune response (see p. 536 Evasion of the host defence line 16–34 and Tissue damage line 38–61).

Evasion of the Host Defence

The host response to anaerobic infection includes phagocytosis and opsonisation and killing by serum immunoglobulin and complement. The virulent species of Gram-negative anaerobe have various means of avoiding and resisting these defence mechanisms. *Bacteroides fragilis* and some black-pigmented, Gram-negative anaerobes produce various extracellular polysaccharides that may form encapsulating structures, not all which are visible by light microscopy (see p. 535 Surface Variation line 21–24). In *B. fragilis* some of these various components have been implicated in adhesion (Patrick *et al.*, 1988), protection against phagocytosis, resistance to killing mediated by the alternative complement pathway (Reid and Patrick, 1984) and the induction of abscess formation (Tzianabos *et al.*, 1993).

Bacteroides fragilis also inhibits macrophage migration and impairs the phagocytosis of other species involved in the polymicrobial infections. Succinic and other short-chain fatty acid metabolic products have also been shown to inhibit chemotaxis, phagocytosis and intracellular killing by phagocytic cells (Rotstein *et al.*, 1989). *Porphyromonas gingivalis* (Laine and von Winkelhoff, 1998) also produces a capsule that protects against phagocytosis and intracellular killing and generates metabolic products that compete with chemotactic peptides, heat-labile opsonins and complement components to block chemotactic receptors on polymorphs. Abscess-derived neutrophils are reported to harbour viable bacteria and be less efficient at killing *B. fragilis* than neutrophils derived from either peritoneal aspirates or peripheral blood (Finlay-Jones *et al.*, 1991). The potential for excreted extracellular polysaccharide and extracellular outer membrane vesicles to mop up opsonin, activate complement and interact with phagocytes, thus diverting the host defence from the bacterial cell, should not be overlooked. Proteases that degrade cytokines, produced by for example *P. gingivalis*, may also interfere with the immune response including phagocytic function.

Fusobacterium necrophorum isolated from animal infection secrete a well-characterised leukotoxin with specific toxicity for cattle and sheep neutrophils (Narayanan *et al.*, 2002); however, it is not clear whether isolates from human infections secrete similar molecules with specificity for human neutrophils.

Several virulent anaerobic species generate products that inhibit or destroy the humoral components of the host's defences. The black-pigmented species produce proteolytic enzymes active against immunoglobulins and complement and most anaerobic bacteria produce soluble metabolites that are leukotoxic, inhibit chemotaxis and damage mucosal cells.

Neuraminidases, which cleave sialic acid from oligosaccharides on host cell glycoproteins and glycolipids, produced by *B. fragilis* could play a key role in virulence (Godoy *et al.*, 1993). There are more than 20 known naturally occurring sialic acids formed by various substitutions and additions to neuraminic acid (Schauer, 1985). There is growing evidence that these sugar residues are involved in the biological activities of the host cells and molecules, in particular in relation to immune system function.

Tissue Damage

Once infection is established, several virulence factors appear to act in combination to produce damage that is manifest as tissue necrosis and abscess formation. Some products of anaerobic metabolism are toxic to mammalian cells: volatile fatty acids and sulphur compounds (e.g. H_2S and amines). Most pathogenic anaerobes also produce extracellular enzymes that hydrolyse tissue components and are thought to play a significant role in pathogenesis (e.g. Rudek and Haque, 1976). *Bacteroides fragilis*, *P. gingivalis*, *P. asaccharolytica*, *Pr. intermedia*, *Pr. melaninogenica* and *Pr. denticola* produce hyaluronidase, condroitin sulphatase, heparinase and a range of enzymes that hydrolyse carbohydrates. All of these enzymes play dual roles, causing tissue damage and providing nutrients for the infecting microbes. *Fusobacterium necrophorum* produces membrane-damaging lipases that may be related to haemolytic activity (Hagelskjaer Kristensen and Prag, 2000).

Proteolytic activity is associated with the strongly proteolytic *Porphyromonas* spp. and some *Prevotella* spp. Proteases, such as the trypsin-like cysteine proteases (gingipains) of *P. gingivalis* (Curtis *et al.* 2001), are thought to be important in the destruction of gingival tissue and of the collagen bridges in the gingival crevice in periodontal disease. Proteases may also be important in the contribution of *P. asaccharolytica* and *'B.' ureolyticus* to tissue damage in ulcerative and gangrenous lesions such as genital and perineal ulcers, decubitus and varicose lesions and diabetic gangrene.

Systemic Inflammatory Response Syndrome

Systemic inflammatory response syndrome (SIRS) or 'sepsis' is the most common cause of death in patients who are critically ill in

non-coronary intensive care units. Endotoxin, containing lipopolysaccharide (LPS), is one bacterial component that is known to play a central role. In patients where there is no obvious bacterial septicaemia, it is thought that the source of the endotoxin is the normal intestinal microbiota. *Bacteroides fragilis* LPS is 5000-fold less toxic in a mouse lethality model than enterobacterial LPS, but given the high numbers of *Bacteroides* spp. in the intestinal tract, where enterobacteria may be outnumbered by up to 1000–1, even allowing for the lower toxicity, it is likely that the numerical dominance of *Bacteroides* LPS means that it plays some role in endotoxic shock and SIRS (Delahooke, Barclay and Poxton, 1995a, b). LPS triggers inflammatory events via the Toll-like receptors (TLRs). LPS from *E. coli* and *Salmonella* spp. interacts with TLR4, whereas LPS from *P. gingivalis* interacts with TLR2, resulting in a less efficient production of proinflammatory cytokines (Erridge, Bennett-Guerrero and Poxton, 2002; Netea *et al.*, 2002). It is now known that the mutation in C3H/HeJ mice that are hyporesponsive to enteric LPS but react with *Bacteroides* spp. LPS (Delahooke, Barclay and Poxton, 1995b) is in the TLR4 gene. The differences in the reactivity of the different LPS molecules appear to relate to their chemical composition, the number, length and composition of the fatty acid chains (or acyl groups) attached to the diglucosamine backbone of the LPSs being different. The lipid A of *P. gingivalis* is less conical in shape than that of *E. coli*. In addition, enterobacterial lipid A diglucosamine is bisphosphorylated whereas in *B. fragilis* it is monophosphorylated; the distal glucosamine residue of the lipid A molecule is lacking in a phosphate group. Also, the core polysaccharide sugar keto-deoxyoctonate (KDO) is phosphorylated which renders it undetectable in the standard KDO thiobarbituric acid assay (Beckmann *et al.*, 1989; Fujiwara *et al.*, 1990).

Whether or not the LPS of *Bacteroides* spp. possesses or is lacking in an O-antigen similar to that found in the enterobacteria is subject to controversy. In the literature, there are reports that extended repeating O-antigen is absent in *Bacteroides* (Lindberg *et al.*, 1990; Comstock *et al.*, 1999). The suggestion is that LPS is more like the lipo-oligosaccharides of, for example, *Nesseria meningitidis* (Jennings *et al.*, 1999) or 'rough' type mutants of the enterobacteria. Other publications, however, quite clearly illustrate silver-stained LPS polyacrylamide gel electrophoresis (PAGE) profiles with ladder patterns characteristic of smooth LPS in *B. fragilis* (Poxton and Brown, 1986) and *B. vulgatus* (Delahooke, Barclay and Poxton, 1995a). The controversy over the presence or absence of an 'O-antigen' component in *Bacteroides* spp. can be explained by the use of different chemical extraction procedures by different laboratories and the variation in the expression of the polysaccharide biosynthesis operons. The *in silico* identity of the genes within the polysaccharide biosynthesis operons, which are controlled by invertible promoters (see p. 535 Surface Variation line 26-28), suggests that some of these are related to the O-antigens of other bacteria (Cerdeno-Tarraga *et al.*, 2005). The LPS of *P. gingivalis* appears to play a significant role in the pathogenesis of periodontal disease, in which (like the LPS of *B. fragilis*) it reduces the opsonic activity of serum, stimulates gingival inflammation, increases the secretion of collagenase from host cells and reduces collagen formation, and induces localised bone resorption around the tooth root. These effects are attributable to the release of biologically active agents, including the cytokines interleukin-1 and tumour necrosis factor from host cells.

Fusobacterium necrophorum is an exception, as its LPS contains classical KDO and displays endotoxic activity similar to that of enterobacterial LPS, particularly during the septicaemic phase of human necrobacillosis.

Enterotoxin

Enterotoxigenic *B. fragilis* were first described as a cause of acute watery diarrhoeal disease in animals and subsequently humans, in particular children (Myers *et al.*, 1987). The enterotoxin is a zinc-dependent metalloprotease of approximately 20 kDa. The possible relationship of the enterotoxin to the virulence of *B. fragilis* in other types of infection remains to be proven as only between 9 and 26% of isolates harbour the gene encoding the toxin (Patrick, 2002).

CLINICAL FEATURES OF ANAEROBIC INFECTIONS

Infections with Gram-negative anaerobes are principally endogenous in source and related to the body sites where these microbes are part of the normal microbiota. Several features are typical of most anaerobic infections: they are necrotising or gangrenous conditions in tissues rendered susceptible by trauma, reduced blood supply and poor oxygenation, often in the presence of a foreign body or blood clot, and producing copious amounts of foul-smelling pus; thrombophlebitis of surrounding blood vessels is common, enhancing the anaerobic conditions. The characteristic smell of anaerobic infections is caused by the volatile end products of anaerobic metabolism. Infections are rarely 'pure', i.e. monobacterial. Anaerobic species are usually present in association with other anaerobic or facultative species in synergic mixtures (see p. 534 pathogenicity line 17–19), but the anaerobes appear to be the principal causes of tissue damage and abscess formation in these mixed infections. From a clinical perspective, infections may be divided into five broad groups: 1. those derived from the gastrointestinal tract, 2. genito-urinary infections in men and women, 3. infections of the head and neck related to the oral microbiota, 4. infections of other soft tissues, and 5. bacteraemia (Finegold and George, 1989; Duerden, 1990).

Gut-Associated Infections

Bacteroides spp. represent a large proportion of the normal microbiota of the lower intestinal tract and are the main cause of serious sepsis associated with surgery, injury, perforation or other underlying abnormality of the large intestine. *Bacteroides* spp. are the main components of the microbiota of post-operative abdominal wound infections, peritonitis and intra-abdominal abscesses (appendix, diverticular or paracolic, pelvic, sub-phrenic etc.). *Bacteroides fragilis* is the most common species isolated from these infections, representing about 75% of the *Bacteroides* isolates in contrast with between 4 and 13% of *Bacteroides* isolates from the faecal microbiota. Thus, *B. fragilis* clearly appears to have particular pathogenic potential and a much greater capacity to cause infection than the other *Bacteroides* spp., as described in the pathogenicity. Amongst the remaining 25% of isolates, *B. thetaiotaomicron* is the most common, with smaller numbers of *B. distasonis* and *B. vulgatus* (Patrick, 2002). The species other than *B. fragilis* are usually found in mixtures of multiple anaerobic species, often representing gross faecal soiling of the tissues. *Bilophila wadsworthia* is associated with gangrenous appendicitis and other intra-abdominal sepsis.

Gram-negative anaerobes are predominant components of the mixed microbiota of peri-anal, pilonidal and perineal abscesses (Duerden, 1991). *Bacteroides fragilis* is again the most common species isolated, but *P. asaccharolytica* and *'B.' ureolyticus* appear more frequently in these infections than in abdominal infections, as they do in other superficial soft-tissue infections such as sebaceous abscesses and decubitus or diabetic ulcers (Duerden, Bennett and Faulkner, 1982) (see p. 539 Superficial soft-tissue infections).

Liver abscesses form a specific clinical group of intra-abdominal abscesses. They are generally polymicrobial and anaerobes, particularly *Bacteroides* spp. and anaerobic cocci often predominate. The route of infection is usually via the portal venous system, and the common underlying causes are colorectal malignancy, ulcerative colitis, Crohn's disease and other intra-peritoneal abscesses. The most common species isolated is, again, *B. fragilis*, and *Bacteroides* bacteraemia is a well-recognised complication. Anaerobes are responsible for only a minority of biliary tract infections, being restricted mainly to obstructive empyema of the gall bladder.

Commonly associated facultative bacteria include *E. coli* and *Streptococcus* spp., in particular *Strep. milleri*, which forms pinpoint colonies and with a characteristic smell.

Genito-Urinary Infections

Gram-negative anaerobes are significant pathogens in a wide range of infections of the genito-urinary tract in both men and women (Duerden, 1991). In men, the infections are principally ulcers and abscesses of the external genitalia and perineum, but in women there are also infections of the vagina and of the uterus and deep pelvic tissues.

In local abscesses associated with the secretory glands in women, e.g. Bartholin's and Skein's abscesses, obstruction of the ducts leads to a build up of secretions and infection with a mixed bacterial microbiota, predominantly anaerobic, that is similar to the microbiota of sebaceous cysts and pilonidal abscesses; *P. asaccharolytica* is a common and important isolate. Anaerobes are also frequent isolates from genital ulcers, a common problem in both men and women. They are probably not primary causes (genital herpes or trauma are common primary diagnoses) but whatever the cause of the initial damage, anaerobes form a major part of the microbiota of established ulcers and contribute to the progressive tissue damage. The term genital ulceration covers a range of superficial necrotising conditions, from erosive balanitis/balanoposthitis in men and superficial labial ulceration in women, through deep, spreading ulcers, typically with undermined edges, to the more severe forms of synergic gangrene. In all groups, regardless of initiating factors, anaerobic bacteria are the predominant cultivable bacteria once the superficial debris is removed. Gram-negative anaerobes are not normally present on the external genitalia but are the most common isolates from infected ulcers. Although *Bacteroides* spp. may be present in some cases, the most common apparently significant isolates are the asaccharolytic and strongly proteolytic *P. asaccharolytica* and *'B.' ureolyticus* which have a particular association with superficial necrotising and ulcerative lesions at various body sites. *Prevotella* spp., principally *Pr. intermedia* (which appears to be the most virulent of the *Prevotella* spp.), are also common isolates – being less common than *P. asaccharolytica* in men but about equally common in women, in whom *Prevotella* spp. are part of the normal vaginal microbiota (Masfari, Kinghorn and Duerden, 1983; Masfari, Duerden and Kinghorn, 1986).

Vaginal discharge is a common complaint of women attending Genito-urinary Medicine clinics and anaerobes are involved in many cases (Duerden, 1991). The most common condition is bacterial (anaerobic) vaginosis in which a disturbance of the normal vaginal microbiota with the loss of predominant lactobacilli and their replacement by increased numbers of Gram-negative anaerobes results in a discharge with an offensive, fishy smell. With the proliferation of *Prevotella* spp., *Gardnerella vaginalis* and *Mobiluncus* spp., the vaginal pH rises, the lactate concentration falls and the amounts of succinate, acetate, propionate and butyrate increase, together with volatile amines that cause the smell. These same metabolites may induce the excessive secretion from the vaginal mucosa, but the factors that initiate the condition, other than its clear association with sexual activity and a relationship with the presence of seminal fluid in the vagina, are still not clear. The other main causes of vaginal discharge – gonorrhoea, chlamydia and trichomonas infection – also cause a major disturbance of the normal microbiota with significant increases in the numbers of Gram-negative anaerobes (Masfari, Duerden and Kinghorn, 1986).

Ascending infections of the uterus and pelvis with vaginal anaerobes (mainly *Prevotella* spp.) or with *B. fragilis* or *P. asaccharolytica* are the cause of serious gynaecological sepsis. Some of these infections are the classical infective complications of pregnancy, parturition or abortion such as post-partum or post-abortal uterine infections in which retained products of conception provide ideal conditions for anaerobes to proliferate. With the control of streptococcal puerperal infection, anaerobes have become the most common cause of post-partum sepsis. The same microbes also contribute to deep pelvic infections unrelated to pregnancy – endometritis, parametritis, tubo-ovarian and pelvic abscesses, generally grouped together as pelvic inflammatory disease – and wound infections and abscesses complicating gynaecological surgery. Laboratory confirmation of the cause of pelvic sepsis is difficult without surgical exploration; laparoscopy does not provide adequate samples for bacteriological culture but evidence from cases that have required open surgery supports the role of anaerobes in these infections (Kinghorn, Duerden and Hafiz, 1986). In gynaecological surgery, the underlying pathology has often resulted in colonisation of the usually sterile deep sites and the incidence of wound infection, before antibiotic prophylaxis was routine, was about 20% and most were anaerobic, with both the vaginal *Prevotella* spp. and *B. fragilis* being common isolates.

Fusobacterium nucleatum, along with *Mobiluncus curtisii*, has been recovered from the amniotic fluid of women with preterm delivery (Citron, 2002).

Infections with Oral Anaerobes

The Gram-negative anaerobes that are part of the normal microbiota of the gingival crevice cause suppurative infections of the gingiva and immediate surrounding tissues related to dental problems but can also cause various abscesses and soft-tissue infections throughout the head, neck and chest (Hardie, 1991).

Gingivitis and periodontal disease are probably the most common anaerobic infections, if not the most common of all infections worldwide, and are the most important cause of tooth loss. Acute ulcerative gingivitis (also known as Vincent's angina, Plaut-Vincent's infection or trench mouth) was one of the first anaerobic infections of man to be recognised. It is associated with poor oral hygiene, malnutrition and general debility and is now seen as a complication of AIDS. Clinically, there is pain, haemorrhage, inflammation and destruction of gum tissue and a foul odour. Spirochaetes and fusiform organisms are seen readily in stained films of the exudate and they may play a role in the disease, but the oral Gram-negative anaerobes, principally *Prevotella* and *Porphyromonas* spp., are also present in large numbers and may be more important in the pathogenesis of the condition. In other forms of periodontal disease, *P. gingivalis* and *Pr. intermedia* have been implicated as significant pathogens in rapidly progressive disease. *Fusobacterium nucleatum* and non-pigmented *Prevotella* spp. are also present in large numbers in periodontal disease and may have a pathogenic role (Tanner and Stillman, 1993).

The oral anaerobes are also important in dental abscesses, root canal infections (a particular association of *P. endodontalis*) and soft-tissue abscesses, e.g. buccal and pharyngeal abscesses. In the normal healthy state, these anaerobes do not colonise other sites in the head and neck and do not cause acute primary infections of the throat, middle ear, mastoid or sinuses. However, when pre-existing damage or prolonged infection leads to the development of chronic otitis media, mastoiditis or sinusitis, these compromised air passages become infected with a mixture of oral streptococci and anaerobes such as *Prevotella* spp. These anaerobes are also the most common cause of brain abscess, especially those originating from chronic otitis media, mastoiditis or sinusitis; cholesteatoma is an important predisposing factor. Infection spreads by direct extension, often with localised osteomyelitis and thrombosis of the lateral sinus. Dental infection may also give rise to brain abscess either by direct extension or by haematogenous spread (Ingham and Sisson, 1991). Similarly, the oral anaerobes can cause lung abscesses as a result of aspiration of organisms from the mouth when there is already some abnormality such as obstruction due to a malignant tumour or an inhaled foreign body (Civen *et al.*, 1995).

Fusobacterium necrophorum subsp. *necrophorum* (previously *F. necrophorum* Biovar A) differs from almost all other Gram-negative anaerobes in being a primary pathogen capable of causing disease in previously healthy people, although there may be synergic involvement of other viruses or bacteria that cause pharyngitis in the initial invasion of the mucosa (Hagelskjaer Kristensen and Prag, 2000).

Epstein–Barr virus infection (infectious mononucleosis) may be a predisposing factor.

Classically, the infection (necrobacillosis or Lemmiere's syndrome) begins as a suppurative tonsillitis with pus formation and a pseudomembrane, which spreads to involve the local lymph nodes in the neck and from there to invade the bloodstream causing septicaemia and disseminating the organisms widely in the tissues to form multiple soft-tissue abscesses at many sites in the body (liver, lung, kidney etc.). It may also occur in children with otitis media, adults with tooth infections and spread from other foci such as sinusitis or mastoiditis in all ages. *Fusobacterium necrophorum* may also cause necrobacillosis arising from sites other than the head and neck in elderly patients with other predisposing disease. In patients with anaerobic sepsis arising from the oropharynx, *F. necrophorum* should immediately be considered as a potential cause, as it is the most frequently isolated species. The classical symptoms of Lemmiere's syndrome are oropharyngeal pain, neck swelling, pulmonary symptoms, arthralgia and fever. It should be considered in patients with severe sepsis and pulmonary symptoms after acute pharyngotonsillar infection. Unilateral suppurative thrombophlebitis of the internal jugular vein is characteristic and can be diagnosed by ultrasonography, axial CT scan or magnetic resonance angiography of the neck. Multiple bilateral necrotic infiltration of the lungs with pleural effusion, empyema and/or pulmonary abscesses is reported in up to 85% of cases (Hagelskjaer Kristensen and Prag, 2000).

Lemmiere's syndrome was first described in the pre-antibiotic era. It became very uncommon during the second half of the 20th century, possibly due to widespread and indiscriminate use of antibiotics for upper respiratory tract infections, but increasing numbers of cases have been reported in the United Kingdom during the past decade (Brazier *et al.*, 2002) and also recently in the United States (Ramirez *et al.*, 2003).

Bone and Joint Infection

Anaerobic bacteria, often along with facultative species, may be associated with both osteomyelitis and septic arthritis. *Bacteroides fragilis* was the predominant anaerobic isolate from osteomyelitis in a 10-year study. In addition, *Prevotella*, *Porphyromonas*, *Fusobacterium* and other *Bacteroides* spp. were isolated. *Bacteroides fragilis* was associated most frequently with infections of the hands and feet where there was underlying vascular disease or neuropathy, whereas *Prevotella*, *Porphyromonas* and *Fusobacterium* were more frequently recovered from skull and bite infections (Brook and Frazier, 1993). Gram-negative non-sporing anaerobes are rarely isolated from prosthetic joint-associated infection, where *Propionibacterium acnes* is the most frequently isolated anaerobe (Tunney *et al.*, 1998). *Bacteroides fragilis* and *Fusobacterium* spp., however, may cause septic arthritis. In approximately 50% of instances this arises from haematogenous spread from a distant focus of infection.

Superficial Soft-Tissue Infections

As well as infections related to the normal carriage sites of Gram-negative anaerobes, another group of soft-tissue infections occurs at sites not immediately adjacent to the mucosae but in tissue damaged by trauma or hypoxia, or where secretory glands become blocked. These infections include sebaceous and pilonidal abscesses, breast abscesses in non-lactating women and infection secondary to inadequate perfusion and oxygenation of the skin and subcutaneous tissues – decubitus and varicose ulcers, diabetic gangrene etc. (Adriaans, Drasar and Duerden, 1991). Although a wide range of anaerobes and facultative species can be isolated from these lesions, there is a particular association between progressively destructive infections and the presence of *P. asaccharolytica* and/or *'B.' ureolyticus* (Duerden, Bennett and Faulkner, 1982). There is a specific association of *F. ulcerans* with tropical ulcers – deep eroding ulcers, usually of the lower limb, occurring across many tropical regions (Adriaans Hay and Drasar, 1987; Adriaans *et al.*, 1987). Since the initial description of this bacterium, further isolates have not been reported. This may have arisen from a phenotypic similarity to *F. varium* (a gastrointestinal species). Many commercial identification kits lack a test for nitrate reduction, a distinguishing feature of *F. ulcerans*. Therefore, an incorrect identification of *F. varium* is generated by the kit database (Citron, 2002).

Bacteraemia

Primary bacteraemia with Gram-negative anaerobes is extremely rare. It is an integral part of necrobacillosis in which *F. necrophorum* invades the bloodstream as part of the spread of infection from the primary site in the throat (see p. 538 Infections with oral anaerobes line 3–5). In most other cases, the bacteraemia is secondary to localised infections at some site in the body, e.g. intra-abdominal abscess and pelvic sepsis, may be the first indication of some serious underlying condition such as a brain abscess (Goldstein, 1996). Bacteraemia with a *Bacteroides* spp. may be the first indication of a malignant tumour of the colon, rectum or cervix. The most common anaerobic species isolated from blood cultures is *B. fragilis*. Mortality and morbidity of *Bacteroides* bacteraemia depends generally upon the underlying cause. A matched-pair study in the United States reported a potential mortality of up to 19% for *B. fragilis* group bacteraemia (Redondo *et al.*, 1995).

LABORATORY DIAGNOSES

The site and nature of the infection (e.g. an abdominal abscess or a necrotising or gangrenous ulcer) may give a clear indication that anaerobes are likely to be involved, and the presence of a foul-smelling exudate or pus is the strongest clinical indication of an anaerobic infection. The diagnosis is confirmed by careful anaerobic culture methods and the identification of the isolates obtained. Because these infections are often associated with sites that have a complex normal bacterial microbiota (often including the organisms that may be the putative pathogens), and because anaerobes are, by their nature, more or less susceptible to exposure to oxygen, care is needed in specimen collection and transport to try to ensure that the organisms sought do not die in transit to the laboratory and that any cultures obtained represent the microbiota at the infected site and not contamination from the normal mucosal microbiota (Drasar and Duerden, 1991; Summanen *et al.*, 1993). Information on all aspects of laboratory diagnosis of anaerobic bacteria and detailed identification protocols can be found in the Wadsworth-KTL Anaerobic Bacteriology Manual (Jousimies-Somer *et al.*, 2002).

Specimen

The most reliable specimens are pus or exudates from the depths of an open lesion or a closed lesion. Specimens such as sputum collected from sites with a normal mucosal microbiota are not suitable for anaerobic culture. Swabs used traditionally to sample wounds and exudates are not ideal for anaerobes that suffer from desiccation and exposure to oxygen, as well as being entrapped in the interstices of the swab. Whenever possible, pus should be aspirated into a sterile container and delivered promptly to the laboratory (Finegold, 1995).

Transport

Direct plating of a sample at the patient's bedside and immediate anaerobic incubation are likely to give the best results, but this is an unattainable ideal in most clinical situations. If pus, or a piece of necrotic tissue obtained by biopsy, is the specimen, it should be transported to the laboratory without delay in a sterile container. Sealed containers with an oxygen-free atmosphere, or from which oxygen can be removed by a simple chemical reaction, are available for optimal transport of such specimens. Pus is generally regarded as

its own best transport medium. If there is no alternative to the use of swabs, these should be placed in semi-solid anaerobic transport medium to prevent both desiccation and the toxic effects of oxygen.

Laboratory Examination

Direct microscopy of pus samples may be helpful in some cases; most of the Gram-negative anaerobes are small, pleomorphic organisms that are difficult to see in direct smears. The most useful immediate examination is direct GLC to detect the presence of the volatile fatty acid products of anaerobic metabolism (Jousimies-Somer *et al.*, 2002). This will not, generally, give any indication of the specific identity of the anaerobes present, but the demonstration of a mixture of short-chain fatty acids confirms the presence of anaerobic bacteria.

Isolation of Gram-negative anaerobes is by anaerobic culture on selective and non-selective media. Anaerobic culture conditions can be provided in sealed anaerobic chambers equipped with air locks and functioning as anaerobic incubators as well as workstations, or in anaerobic jars. The anaerobic atmosphere generally used in cabinets is N_2 80%, H_2 10% and CO_2 10%, as the growth of many anaerobes is stimulated by CO_2. The same gas mixture can be used in jars operated by an evacuation and refill system and equipped with room-temperature-active catalysts (e.g. palladium) to remove any remaining oxygen. Alternatively, jars can be used with sachets that generate H_2 and CO_2 when water is added and depend upon the H_2 to remove all the oxygen from the jar with the help of the catalyst. There are many variations on the basic anaerobic jar methodology but all must be carefully controlled to ensure that as much as possible of the O_2 is removed and that air cannot leak back into the system.

Many anaerobes grow more slowly than most common aerobic pathogens, and anaerobic cultures must be incubated for at least 48 h without exposure to oxygen and for an overall minimum of 72–96 h; however, some of the more virulent anaerobes (e.g. *Clostridium perfringens* and *B. fragilis*) produce acceptable growth in 24 h, and most laboratories would not wish to delay the diagnosis by 24 h. If the use of duplicate sets of plates where anaerobic jars are in use (one for examination after 24 h and the other to remain undisturbed for 72–96 h) is considered too extravagant of resources, a useful compromise is for the one set of plates to be examined quickly after 24 h and then re-incubated after as little exposure to air as possible for another undisturbed 48–72 h. This difficulty is overcome by the use of anaerobic cabinets in which the initial examination can be done without removing the plates from the anaerobic atmosphere.

Various media have been recommended for the optimal growth of Gram-negative anaerobes. All comprise a rich nutrient base with added blood. *Bacteroides fragilis* grows well on most formulations of blood agar, but better growth of a wider range of more fastidious species needs more specially enriched media. Most species grow more quickly and produce larger colonies on media such as Fastidious Anaerobe Agar, Brucella agar or BM medium with ingredients such as proteose peptone, trypticase and yeast extract. Menadione and haemin enhance the growth of some species, especially of *Prevotella*; other demanding strains such as *'B.' ureolyticus* require formate and fumarate for good growth and *Bil. wadsworthia* has an absolute requirement for pyruvate. The use of lysed blood assists in the early recognition of pigment production.

As anaerobic bacteria are generally present in mixed infections, their isolation is aided by the use of selective media containing antibiotics inactive against anaerobes. Aminoglycosides have been widely used for many years. The neomycin blood agar developed for the selective isolation of clostridia is too inhibitory for many Gram-negative anaerobes, and kanamycin (75 mg/l) gives better results. An alternative that may be preferred is the use of nalidixic acid (10 mg/l). When it is desirable to eliminate Gram-positive bacteria, the addition of vancomycin (2.5 mg/l) provides a medium highly selective for *Bacteroides* spp. Selective media should never be used without parallel non-selective cultures and growth on the selective media generally requires extended incubation.

Identification of Gram-Negative Anaerobes

Detailed identification to species level of all isolates of Gram-negative anaerobes is beyond the scope of most diagnostic laboratories and would require excessive commitment of resources. They are significant pathogens, however, and certain species either show greater virulence than most of their group (e.g. *B. fragilis*) or are associated with particular types of infections (e.g. *F. necrophorum*, *P. gingivalis* and *P. asaccharolytica*). It is important that primary diagnostic laboratories can at least determine the presence of the main pathogens and identify other significant isolates at least to genus level, for more detailed study in a reference or research laboratory when appropriate.

Preliminary allocation of isolates to the main genera can be made by a relatively simple set of tests based upon colonial and microscopic morphology, growth in the presence of bile (20% in broth or agar medium) or sodium taurocholate (disc tolerance test), disc resistance tests with antibiotics (neomycin 1 mg, kanamycin 1 mg, penicillin 1 or 2 units, rifampicin 15 µg, colistin 10 µg and vancomycin 5 µg) catalase and urease production (Table 45.4), supported by GLC if available.

More detailed identification depends upon sets of biochemical tests with carbohydrate and other substrates and detection of particular enzyme activities. Some of these can be performed with commercial kits (especially the enzymes tests), and these can be useful for the identification of the more common species isolated from clinical specimens. However, most of these systems suffer from the attempt by most manufacturers to produce a single kit for the whole range of anaerobes – Gram-positive and Gram-negative, cocci and bacilli. Tables 45.5–45.9 summarize the sets of tests that enable the identification of most clinically significant Gram-negative anaerobes. The

Table 45.5 Preliminary identification of Gram-negative anaerobic bacteria of clinical importance

	Bacteroides	*Bilophila*	*Prevotella*	*Porphyromonas*	*Fusobacterium*	*Leptotrichia*	*'B.' ureolyticus*
Growth in bile (20%)	+	+	–	–	+/–	+/–	–
Tolerance of taurocholate	+	+	–	–	+/–	+/–	–
Antibiotic disk resistance tests							
Neomycin (1 mg)	R	S	S	S	S	S	S
Kanamycin (1 mg)	R	S	R	R	S	S	S
Penicillin (1–2 units)	R	R	S/R	S/R	S	S	S
Rifampicin (15 µg)	S	S	S	S	R/S	S	R
Colistin (10 µg)	R	S	S/R	R	S	R	S
Vancomycin (5 µg)	R	R	R	S	R	S	R
Urease	–	+	–	–	–	–	+
GLC	Ac, Su	Ac, Su	Ac, Su	*n*-Bu	*n*-Bu	Pro, Lac	Ac, Pro

Ac, acetic; Lac, lactic; *n*-Bu, *n*-butyric; Pro, propionic; Su, succinic.

Table 45.6 Identification of *Bacteroides* spp.

	B. fragilis	*B. vulgatus*	*B. distasonis*	*B. merdae*	*B. caccae*	*B. ovatus*	*B. thetaiotaomicron*	*B. eggerthii*	*B. uniformis*	*B. stercoris*	*B. splanchnicus*
Indole	−	−	−	−	−	+	+	+	+	+	+
Aesculin hydrolysis	+	−/+	+	+	+	+	+	+	+	+	+
Fermentation of											
glucose	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	−
sucrose	+	+	+	+	+	+	+	−	+	+	−
rhamnose	−	+	+/−	+	+	+	+	+	−	+	−
trehalose	−	−	+	+	+	+	+	−	−	−	−
mannitol	−	−	−	−	−	+	−	−	−	−	−
arabinose	−	+	−	−	+	+	+	+	+	−	+
salicin	−	−	+	+	−	+	+	+	−	−	+
xylan	−	−	−	−	−	+	−	+	+/−	+/−	−
α-fucosidase	+	+	−	−	+	+	+	−	+	+/−	+

Table 45.7 Identification of *Prevotella* spp.

	Pr. buccae	*Pr. oris*	*Pr. zoogleoformans*	*Pr. heparinolytica*	*Pr. oralis*	*Pr. veroralis*	*Pr. buccalis*	*Pr. oulora*	*Pr. bivia*	*Pr. disiens*	*Pr. melaninogenica*	*Pr. denticola*	*Pr. loescheii*	*Pr. intermedia**	*Pr. nigrescens**	*Pr. corporis*
Pigment	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Indole	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−
Aesculin hydrolysis	+	+	+	+	+	+	+	+/−	−	−	−	+	+/−	−	−	−
Hippurate hydrolysis	−	−	−	−	−	−	+	−	+	+	...	...	...	...	...	...
α-Fucosidase	−	+	+	+	+	+	+	−	+	−	+	+	+	+	+	−
Fermentation of																
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−
lactose	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−	−
xylose	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
arabinose	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
cellobiose	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	−
salicin	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
xylan	+	−	−	−	−	−	−	−	−	−	...	...	...	...	...	...
inulin	+	−	+	+/−	−	+	+/−	−	−	−	...	...	...	...	...	...

The only phenotypic distinguishing test is multi-focus enzyme electrophoresis for glutamate and malate dehydrogenases; *Pr. nigrescens* has slower mobilities with both enzymes.

Table 45.8 Identification of pigmented human strains of *P.* spp.

	P. asaccharolytica	*P. gingivalis*	*P. endodontalis*
Pigment	+	+	+
Indole	+	+	+
Trypsin-like activity	–	+	–
Phenylacetic acid production	–	+	–
α-Fucosidase	+	–	–

media and methods for performing these tests are varied, and details may be found in manuals or monographs devoted specifically to anaerobic microbiology – the Wadsworth Anaerobic Bacteriology Manual sixth edition (Jouseimies-Somer *et al.*, 2002), the Virginia Polytechnic Institute Anaerobe Laboratory Manual (Moore and Moore, 1977) or Anaerobes in Human Disease (Wren, 1991). Baron and Citron (1997) provide an outline of a cost-effective protocol.

Non-Culture Detection Methods

The ability to identify reliably obligately anaerobic bacteria directly in clinical samples without the need for culture is particularly attractive. Despite the best efforts of clinical bacteriologists to ensure the handling and transit of samples under anaerobic conditions, a high proportion of infections may be missed as a result of loss of viability (Patrick *et al.*, 1995).

Techniques have been developed in some research laboratories for the direct detection of specific pathogenic anaerobes (*B. fragilis*, *P. asaccharolytica*, *P. gingivalis*, *Pr. intermedia* and *Bil. wadsworthia*) by DNA probes and by polymerase chain reaction (PCR) technology, but as yet, there are no non-culture detection methods for anaerobes in general use in clinical diagnostic laboratories. PCR finger printing identification (e.g. amplified ribosomal DNA restriction analysis) of pure culture isolates has resulted in good identification and differentiation of *Bacteroides*, *Prevotella and Porphyromonas* spp. of the *fragilis* group, as has a multiplex PCR assay based on 16S rRNA, the 16S–23S rRNA intergenic spacer region and part of the 23S rRNA gene (Liu *et al.*, 2003). PCR-based assays for the detection of enterotoxigenic *B. fragilis* have also proved reliable. Microarrays enabling the simultaneous detection of multiple genes and therefore multiple bacterial species have been applied to studies of the faecal microbiota. Fluorescence *in situ* hybridisation of 16S rRNA is possible with pure cultures of *B. fragilis*; however, such techniques must be approached with caution if they are to be applied directly to clinical samples, as the penetration of the probe into the bacterial cell is dependent on the nature of encapsulating surface structures.

It is likely, however, that accurate and specific multiplex and quantitative (real-time) PCR diagnostic tests or microarray-based technologies will ultimately become part of routine diagnosis once inherent problems of sensitivity in clinical specimens and potential for contamination from exogenous DNA are resolved.

Antibiotic Susceptibility of Gram-Negative Anaerobes

Approaches to susceptibility testing of Gram-negative anaerobes have been debated amongst microbiologists and infectious disease physicians for many years. A major consideration is whether susceptibility testing should be, as for most aerobes, concentrated in the primary laboratories doing individual susceptibility tests on clinical isolates from their patients or whether strains should be collected for batch testing in recognised specialist centres while individual clinical treatment is based upon accumulated data generated by that batch testing. For aerobes, laboratories test their significant isolates and clinicians expect prompt susceptibility data on their own patients' organisms. This has not been the case with anaerobes because of three perceptions: 1. anaerobic investigations are slower – initial culture may take several days and susceptibility tests at least 2 days more, so that individual results are irrelevant to patient management; 2. methods for susceptibility testing of anaerobes are unreliable, especially when performed on an individual basis, and batch testing in specialist centre gives more reliable results; and 3. the susceptibility of anaerobes is predictable so that clinical treatment can be based upon the batch data with confidence.

These premises were the basis for susceptibility testing of anaerobes having been neglected in primary laboratories and concentrated in specialist centres that receive clinical isolates from a wide range of sources. However, there is an increasing view from clinicians and microbiologists that the same standard of laboratory data is required in anaerobic infections as in aerobic infections. Testing of individual isolates is supported by four changed perceptions: 1. antibiotic susceptibility in anaerobes is variable and patterns differ in different places and at different times; 2. modern laboratory methodology should enable the prompt isolation of the more common anaerobic pathogens; 3. methods for susceptibility testing of anaerobes are available that can be used in primary laboratories; and 4. the most important reason, patients managed with access to accurate and specific laboratory data on their own anaerobic isolates recover more rapidly with fewer complications.

Both approaches are necessary. Primary laboratories need to develop adequate susceptibility tests on anaerobes for first-choice agents while reference laboratories should enhance their activities in collecting strains from a wide range of sources and batch testing to provide the best general advice for clinicians in choosing empirical therapy and devising antibiotic policies.

Methods

The selection of methods for susceptibility testing of anaerobes has been a major difficulty. The two basic methodologies for rapidly growing, relatively non-fastidious aerobes – disc susceptibility tests on solid media and breakpoint determination in liquid or solid media – are not readily adaptable to anaerobic work, are difficult to standardise and do not give reliably reproducible results with anaerobes. Anaerobes tend to grow more slowly than many common aerobes, and the balance between antimicrobial effect, bacterial growth and antibiotic degradation varies much more, affecting disc susceptibility methods in particular. Many anaerobes need more enriched media than are used for susceptibility testing of aerobes; they are more difficult to standardise, and there are interactions between media components and the antibiotics. The presence of fermentable nutrients often results in a very significant drop in pH in the test medium which can have a major impact of the results obtained with pH-sensitive antibiotics. Similarly, many anaerobes require CO_2 for growth and all

Table 45.9 Identification of *Fusobacterium* spp.

	F. nucleatum	*F. necrophorum*	*F. pseudonecrophorum*	*F. russi*	*F. naviforme*	*F. mortiferum*	*F. varium*	*F. gonidiaformans*	*F. ulcerans*
Indole	+	+	+	–	+	–	+/–	+	–
Lipase	–	+	–	–	–	–	–/+	–	–
Aesculin hydrolysis	–	–	–	–	–	+	–	–	–
Propionate from									
lactate	–	+	+	–	–	–	–	–	–
threonine	+	+	+	–	–	+	+	+	+

standard anaerobic gas mixtures contain CO_2 which again lowers the pH (Watt and Brown, 1985). Many anaerobes do not grow well from small inocula, and semi-confluent growth is difficult to achieve. A further practical difficulty arises because the MICs of several agents for anaerobes cluster near the recommended breakpoints for the agents, so that minor variations in method and inevitable margins of error in any technique can give variable results in terms of clinical interpretation and recommendations. Therefore, disc susceptibility tests for anaerobes have been regarded as unreliable for other than the fast-growing anaerobes. Recommended methods in the past have been based upon agar dilution with multipoint inoculation or broth micro-dilution or macro-dilution methods. Some of these have not been entirely satisfactory and are not appropriate for testing individual clinical isolates, hence the methodology has exerted pressure in favour of batch testing in reference laboratories. The E test (AB Biodisc, Denmark) provides reliable susceptibility testing with MIC determination for individual isolates. A plastic strip coated with an antibiotic gradient on one side and with an MIC interpretation scale on the other is placed on a seeded plate as in disc susceptibility testing. After incubation, the edge of the zone of inhibition reaches the scale at a point equivalent to the MIC. This shares some of the disadvantages of disc methods, but experience has shown it to be generally reliable for a wide range of anaerobes (Wexler, 1993; Duerden, 1995; Jousimies-Somer *et al.*, 2002).

It is likely that molecular detection methods for antibiotic resistance genes will be incorporated into molecular identification protocols. A PCR-restriction fragment length polymorphism analysis identification method for *Bacteroides* spp. that incorporates *nim* gene detection has proved to be rapid and accurate (Stubbs *et al.*, 2000).

Antibiotic Susceptibility

The susceptibility of Gram-negative anaerobes varies considerably both between and within the major genera. Metronidazole has been the mainstay of therapy and prophylaxis for 20 years, and it is re-assuring that resistance amongst Gram-negative anaerobes has remained relatively low (Dubreuil, 1996; Snydman *et al.*, 1996), so that it remains the drug of choice for many of these infections. Its use has, however, been extended to the treatment of peptic ulcer disease with the recognition of the involvement of *Helicobacter pylori* (Freeman *et al.*, 1997). Whether this wider use of metronidazole is leading to the development of increased resistance in *Bacteroides* of the normal microbiota is not proven, but metronidazole resistance has become more widely recognised in *Bacteroides* spp. in the last 5 years. Of the eight metronidazole resistance genes described (*nimA-G*), all but one (*nimB*) can be found on potentially mobile genetic elements in *Bacteroides* spp. Within the United Kingdom, referral of isolates to the Anaerobe Reference Laboratory has shown the incidence of metronidazole resistance in *Bacteroides* spp. increasing to around 7% since 1995 (Brazier, Stubbs and Duerden, 1999).

A number of different types of mobile genetic elements, including self-transmissible large conjugative transposons and plasmids as well as mobilisable plasmids and transposons, have been identified in the Gram-negative anaerobes (Sebald, 1994; Smith, Tribble and Bayley, 1998). There is evidence of horizontal transfer of antibiotic resistance genes via these mobile elements and the potential transfer of these to facultative species (Vedantam and Hecht, 2003). In the case of the large conjugative transposons that carry a tetracycline resistance gene, exposure to tetracycline, even at sub-inhibitory concentrations, increases the frequency of conjugation (Patrick, 2002). The normal microbiota may therefore represent a potential reservoir of transmissible antibiotic resistance genes.

Bacteroides spp. isolates of *B. fragilis* and other *Bacteroides* spp. are resistant to benzylpenicillin and ampicillin/amoxicillin due to the production of an amp-C type β-lactamase (Wang, Fast and Benkovic, 1999); in almost all cases this resistance is overcome by the use of a combination of ampicillin/amoxicillin with the β-lactamase inhibitors clavulanate or tazobactam. *Bacteroides* spp. are also resistant to many other β-lactam agents. Cefoxitin has been widely used in the United States for anaerobic infections because it has greater activity against *Bacteroides* spp. than most β-lactam agents, but results of susceptibility tests are variable with up to 40% of isolates giving MIC values around or above the recommended breakpoint of 32 mg/l. Most isolates in the United Kingdom remain susceptible to imipenem, but resistance due to the production of a metallo-β-lactamase, not affected by clavulanate or tazobactam, and encoded by the *cfiA* (*ccrA*) gene does occur and may become a more significant problem in the future. With the inhibitors of protein synthesis, many strains are resistant to erythromycin and tetracycline but most are susceptible to clindamycin. Like the *Bacteroides* spp., *Bil. wadsworthia* produces a β-lactamase and is resistant to most β-lactam agents.

Porphyromonas and Prevotella

Although the genera are generally more susceptible to antimicrobial agents than the *Bacteroides* spp., results are still unpredictable. In the past, they were considered to be generally penicillin sensitive, but one-third or more of clinical isolates, especially of *Prevotella* spp., are resistant due to β-lactamase production. Thus, they are susceptible to amoxicillin/ampicillin and clavulanate combinations. Most are susceptible to cefoxitin and highly susceptible to imipenem. They are generally susceptible to erythromycin and clindamycin, but up to half of clinical isolates are resistant to tetracycline and up to 15% are resistant to ciprofloxacin and clindamycin.

Fusobacterium

Most *Fusobacterium* spp. isolates are highly susceptible to a wide range of agents including penicillin and all the β-lactam agents, but resistance mediated by β-lactamase production is increasing. Ceftizoxime, ciprofloxacin and erythromycin are poorly active and up to 30% of strains may be resistant to clindamycin and tetracycline (Finegold and Jousimies-Somer, 1997).

MANAGEMENT OF ANAEROBIC INFECTIONS

Because of the multi-factorial nature of infection with Gram-negative anaerobes (p. 537–539 Clinical features of anaerobic infections) – mixed infections, tissue necrosis, inadequate blood supply/oxygenation, patient already compromised by underlying disease – the management of these infections requires a combination of approaches. Antibiotic treatment is important but is rarely effective if used alone. The choice is generally between metronidazole, a β-lactam agent such as amoxicillin-clavulanate or imipenem, and clindamycin. The recognition of the presence of aerobic or facultative organisms may also need to be addressed either by adding an agent effective against, e.g. *E. coli* to the metronidazole, or by choosing an agent effective against both (e.g. imipenem).

It is axiomatic in medical microbiology that antibiotics alone are ineffective, as treatment for abscesses and many of the anaerobic infections have a component of tissue necrosis and abscess formation. Debridement of necrotic tissue and drainage of pus are essential to the effective management of these infections. An interesting exception is disseminated *F. necrophorum* infection with metastatic abscess formation; this responds well to treatment with penicillin or other antibiotics alone.

Many of the patients who develop anaerobic infections are seriously ill, e.g. peritonitis following major abdominal surgery, and may be developing sepsis syndrome due to the anaerobes or other components

of the mixed infection. These patients clearly need intensive supportive therapy for respiratory and circulatory functions.

PREVENTION AND CONTROL

As most infections with Gram-negative anaerobes are endogenous, cross infection is not a significant problem with these patients, although isolation may be appropriate because of the severity of the infection and, on some occasions, because of the intense malodour created by the anaerobes which can be embarrassing and distressing to the patient and unpleasant and disturbing for others.

Prevention of these infections depends upon prophylactic measures that can be taken to avoid creating the conditions that allow anaerobes to become established. Most of these measures relate to surgical practice in abdominal, gynaecological or oral-facial surgery – i.e. in areas where anaerobes predominate in the normal microbiota. Prevention of post-operative anaerobic infection in these situations depends upon good surgical practice, reduction in the bacterial challenge and the use of appropriate prophylactic antibiotics. Surgical skill goes a long way towards preventing anaerobic infections by not leaving non-viable tissue or foreign bodies at the operation site, ensuring adequate perfusion and oxygenation of the tissues that are left, preventing leakage from the viscus or the accumulation of fluid and closing off any potential spaces where infection can develop. Thus, the 'soil' is not conducive to anaerobic infection. The bacterial challenge can be reduced in part by physical removal of the normal microbiota. This is particularly important in the use of laxatives, enemas or even colonic irrigation to reduce the volume of faeces in the colon before abdominal surgery. A further important prophylactic measure is the use of prophylactic antibiotics per-operatively. These have made a major impact in reducing the incidence of post-operative infection during the last three decades. Since the advent of specific prophylaxis encompassing anaerobic organisms, the incidence of post-operative infection in abdominal and gynaecological surgery has fallen dramatically, e.g. from 35 to 5–10% for major colonic surgery, 20 to <5% for appendicectomy, 25 to 5% for hysterectomy (Willis and Fiddian, 1983). The aim of prophylaxis is to have high levels of appropriate antibiotics in the tissues at the time of the operation to prevent any implanted organisms becoming established. Prophylaxis in this way is not designed to destroy the bacteria in their normal habitat, e.g. in the gastrointestinal lumen. The choice of agent must include effective anti-anaerobe activity – usually by including metronidazole or amoxicillin/clavulanate – plus activity against other organisms likely to be present. Thus, in abdominal surgery, an aminoglycoside (e.g. gentamicin) or a cephalosporin (e.g. cefuroxime or cefotaxime) may be added to the metronidazole. In some circumstances, amoxicillin/clavulanate may fulfil both functions. For effective prophylaxis and to avoid disturbing the normal microbiota or selecting resistant strains before surgery, the antimicrobial agents should be given by intravenous injection at the induction of anaesthesia. For most operations, a single dose at this stage is sufficient to give good protection. In some regimens, a second dose after 4 h or at the completion of the operation has been recommended in more complex and longer operations. In any case, the whole period of prophylaxis should not exceed 24 h. If infection is already present at operation and more prolonged use of antibiotics is clinically necessary, this becomes a therapeutic course for established infection and not a prophylactic measure (Willis, 1991; Keighley, 1992).

Mobiluncus

The genus *Mobiluncus* represents a group of slender, curved, rapidly motile, Gram-variable anaerobic rods found in the vagina and associated with *Gardnerella vaginalis* and *Prevotella* spp. in bacterial (anaerobic) vaginosis (Duerden, 1991). It is a member of the Actinomycetaceae and the phylum of high mol% G+C Gram-positive bacteria, the Actinobacteria, and has a G+C content of 52–56 mol%. Two morphologically distinct types of *Mobiluncus* are recognised and represent two separate species (Tiveljung, Forsum and Monstein, 1996). *Mobiluncus curtisii* strains have short Gram-positive or Gram-variable cells and are much more active in biochemical tests – they hydrolyse arginine and hippurate, reduce nitrate and produce β-galactosidase. *Mobiluncus mulieris* gives negative reactions in these tests and has much longer cells that appear Gram negative. However, both have a cell wall that is essentially of Gram-positive structure, but the peptidoglycan layer is thin, probably resulting in the variable staining pattern. Both species grow well anaerobically but will grow in 5% O_2 and growth is enhanced by CO_2. Although they may be present in large numbers in vaginosis and were described in vaginal discharge by Curtis in 1915, their role in disease is not clear. PCR detection indicates that, whereas *M. curtisii* is frequently associated with bacterial vaginosis, *M. mulieris* may also be detected in apparently healthy women (Schwebke and Lawing, 2001). *Mobiluncus* spp. are always present along with other potential pathogens such as *G. vaginalis* and *B. fragilis* in vaginosis and may have a synergic role (Spiegel, 1987). Treatment with metronidazole removes the anaerobes from the synergic mixture, and vaginosis usually responds well to short-course treatment with agents active against anaerobes, such as metronidazole to which *Mobiluncus* spp. are often resistant (Jones *et al.*, 1985). In addition, as there is a relationship with the deposition of semen in the vagina, the use of a condom will prevent the development of vaginosis (Duerden, 1991).

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Appendix
A

S Patrick
1981
Research Summary Report

Studies on the Pathogenicity of Bacteroides sp.

Sheila Patrick
December, 1981.

INTRODUCTION

Bacteroides sp., in particular B. fragilis, are frequently isolated from intra-abdominal infections, female genital tract infections and bacteremia, (Lindberg et al, 1979) and it has been suggested that serum resistance is one of the major factors contributing to the pathogenicity of this organism, (Casciato, 1979). Different species of Bacteroides vary in their susceptibility to the bactericidal effect of human serum, after in vitro growth, (J.Reid, unpubl.). For example, B. fragilis strains ATCC 23745 and NCTC 10584 are serum resistant and B. ovatus strain ATCC 8483 is serum sensitive.

The presence of a polysaccharide capsule may be an important factor in the virulence of B. fragilis. Indeed, purified capsular material from B. fragilis ATCC 23745 was shown to induce the production of abscesses when implanted in the peritoneum of Wistar rats, (Onderdonk et al., 1977). However, the relationship of the polysaccharide capsule to serum resistance remains unclear. B. ovatus strain ATCC 8483 produces a large capsule, but is serum sensitive.

The encapsulation of a number of Bacteroides cultures was examined by Babb and Cummins (1978). They noted a variation in the percentage of encapsulated cells within an individual culture. This did not vary with the culture age, incubation temperature, pH or the presence of several organic and inorganic nutrients. Instability in the production of capsules in B. thetaiotamicron (a faecal isolate) was noted by Burt et al. (1978). This isolate produced two unstable colonial variants, capsulate and non-capsulate. The change from capsulate to non-capsulate occurred at a rate of $1{\cdot}4 \times 10^{-2}$/cell /generation, at 37°C. Treatment with plasmid curing agents did not affect this frequency. It is interesting to speculate that this phenotypic shift may be similar to the flagellar phase variation found in Salmonella sp. The inversion of a DNA sequence 995 base pairs long controls the expression of two different flagellar antigens and occurs at a rate of between 10^{-3} and 10^{-5}/cell /generation, (Silverman et al., 1981).

The phenotype of a bacterium can also vary considerably with the growth conditions. For example the surface antigens of E. coli varied with the limiting

nutrient when this organism was grown in continuous culture. It is therefore likely that the full range of pathogenic determinants are only expressed when growth occurs *in vivo*, (P. Taylor, unpubl.).

An investigation of the characteristics of *in vivo* grown organisms is therefore of prime importance in studies on the pathogenicity of *Bacteroides sp.*

Preliminary Experiments : Summary of Results

1. ENCAPSULATION

The presence of capsules was demonstrated by both India Ink and Eosin/Carbol Fuchsin staining techniques (Cruickshank, 1965). The capsule was larger when *Bacteroides sp.* were grown in a defined medium (van Tassel and Wilkins, 1978) than when grown in the complex medium of Deacon *et al.* (1978)

Microscopic examination of Eosin/Carbol Fuchsin stained smears showed that *B. fragilis* ATCC23745 produced approximately 20% encapsulated cells and *B. fragilis* NCTC 10584 produced less than 1%. Lindberg *et al* (1979) noted that 77% of *B. fragilis* strains isolated from clinical infections had more than 90% encapsulated cells. However, the loss of surface antigens with continued laboratory sub-culture is relatively common, and possibly occurs as a result of the removal of the selective pressures of *in vivo* growth, (Costerton, 1974).

The two types of bacteria could be seperated by application of broth culture to a Percoll step density gradient (20-80% in 20% steps) and centrifugation in an MSE Superminor bench centrifuge at 4,000 r.p.m. for 20 minutes. The capsulate bacteria remained at the 0 to 20% interface and the non-capsulate banded at the 60 to 80% interface.

Sub-culture of the two bands resulted in the reversion of the non-capsulate to the initial percentage of encapsulation after 24 hours incubation in broth culture. The encapsulated fraction only began to revert after 3 to 4 days of daily sub-culture. The degree of encapsulation was determined purely by microscopic methods. No gross difference in the colony morphology was apparent. Individual colonies contained mixed populations of capsulate and non-capsulate.

2. EXPERIMENTAL INFECTION

Chambers containing live organisms were implanted into the mouse peritoneum according to the method of Day et al (1980).

Preliminary experiments, using high inocula (10^8 to 10^9 colony forming units (c.f.u.)/ml.) were carried out to determine the survival of B. fragilis ATCC 23745 within the chambers.

Bacterial suspensions were incubated for 24 hours within the mouse peritoneum. When the bacteria were suspended in $\frac{1}{4}$ strength Ringer's solution the number of c.f.u./ml. dropped by 2 to 3 logs. However, numbers were maintained at a constant level if cysteine HCl (0·05%) was added.

Future Aims

1. The use of the electron microscope to clearly define the capsule e.g. reaction with anticapsular serum followed by polycationic ferritin labelling (Weiss et al., 1979)

2. The determination of growth/survival of different Bacteroides sp. in peritoneally implanted chambers and the relationship of this to

i. in vitro serum sensitivity

ii. encapsulation.

3. The inclusion of phagocytes in the chambers, and an examination of the effect of this on the survival of Bacteroides sp.

4. If in vivo growth occurs, a comparison with the characteristics, (e.g. morphology serum resistance) of in vitro grown organisms.

5. An investigation of mixed inocula. E. coli is frequently present in the initial stages of anaerobic infections. Therefore it would be of interest to examine the effect of the presence of E. coli either

a. mixed with the Bacteroides in a chamber

or b. in a chamber adjacent to the Bacteroides, seperated by a membrane filter.

6. An investigation of the host's immune response to the presence of Bacteroides in the peritoneum.

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Appendix
B

Post-graduate students whose work, under my supervision at Queen's University Belfast, contributed to the publications in this thesis.

Name	**Degree**	**Year of Graduation**
Lutton DA	PhD	1991
Stewart LD	PhD	1993
Wilson K	MMedSci	1992
McKenna J	MMedSci	1994
Perera R	MMedSci	1997
Hanna D	MMedSci	1997
Ramage G	PhD	1999
Stevenson L	MMedSci	1998
O'Hagan S	MPhil	2001
Glenn JV	PhD	2003
Valanne S	PhD	2003
Connery N	PhD	2003
Douglas L	PhD	2003
McMullan M	MPhil	2004
McLorinan G	MD	2006